Relationships of Selected CYP and *ABCB1* Single Nucleotide Polymorphisms and Patient Characteristics with the Pharmacokinetics of Twice Daily Tacrolimus and Advagraf® in Renal Transplant Recipients

By

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To My Parents, Husband

and My Daughter

Statement of Originality

This is entirely my own work and all the quotations, illustrations and source materials have been appropriately acknowledged.

Toqa El-Nahhas, January 2016

Abstract

Tacrolimus is one of the most potent immunosuppressive agents. It has almost replaced ciclosporin (CsA) as the drug of first choice for the prevention of graft rejection after kidney transplantation (Bouamar et al, 2013). Tacrolimus was initially available as a preparation requiring twice daily administration: Prograf®. A prolonged release preparation, Advagraf®, has become available recently with proven efficacy and safety following once daily dosing. Tacrolimus has a narrow therapeutic window and its high pharmacokinetic variability renders dose selection challenging. Therefore, therapeutic drug monitoring (TDM) is used routinely to direct tacrolimus dosing. To some extent, this variability is influenced by genetic factors. Enzymes in the cytochrome P4503A family (CYP3A) and the drug transporter P-glycoprotein (P-gp) play important roles in the absorption and metabolism of tacrolimus (MacPhee et al, 2002). The influence of the *CYP3A5*3* and *ABCB1 3435C>T* genotypes on the pharmacokinetics of immediate release tacrolimus; Prograf® is well-defined. However, it is unclear for prolonged release tacrolimus; Advagraf®. Recently identified polymorphisms *CYP3A4*22* and P450 Oxidoreductase (*POR*28*) were reported to have additional effects on tacrolimus pharmacokinetics and dose requirement (Jonge et al., 2011; Elens et al., 2013). Recently, 4β-hydroxycholesterol (4β-OHC) has been shown to be an endogenous marker of P450 3A activity in clinical practice (Diczfalusy et al., 2011). Prednisolone is a known inducer of both CYP3A and P-gp. The role of *CYP3A4*22* and *POR*28* in prednisolone metabolism is unknown. An inverse correlation between corticosteroid daily dose and tacrolimus exposure was demonstrated in renal transplant recipients (Anglicheau et al., 2003a). Achieving therapeutic trough concentration is of vital importance during the period immediately after transplantation. Therefore, the identification of parameters predictive of the optimal tacrolimus dosage would be a great clinical asset in the determination of adequate tacrolimus administration. Furthermore, high within-patient variability (WPV) in tacrolimus exposure is considered as a risk factor for allograft loss and late acute rejection (Wu, et al. 2011). The causes of this variability are not completely understood.

The studies outlined in this thesis were carried out on stable renal transplant patients treated with twice daily tacrolimus (Prograf®or Adoport®) and were switched to the same total daily dose of Advagraf®. 24 hours pharmacokinetic profiles were performed before and two weeks after the change. In order to exclude the use of prednisolone as a confounding factor, only patients on not more than 5 mg prednisolone daily were included. The within-patient variability (WPV) was calculated based on the dose-normalized tacrolimus trough blood concentrations (C_0). Analysis of C_0 was also made during periods of stable tacrolimus doses. This study was designed to assess the influence of genetic polymorphisms *CYP3A5*3* and *ABCB1* 3435C >T on tacrolimus pharmacokinetics of immediate- and prolonged- release tacrolimus formulations and their correlation with tacrolimus dosing in 64 stable renal transplant recipients. Genotyping at *CYP3A4*22* and *POR*28* loci in this study was undertaken to ascertain any influence of these genes on the pharmacokinetics of twice and once daily tacrolimus formulations. The influence of switching stable renal transplant patients to once daily tacrolimus formulation (Advagraf®) on WPV was investigated. In a secondary exploratory study to investigate the potential utility of 4β-OHC as a CYP3A biomarker in informing tacrolimus dosing, 4β-OHC concentrations in plasma samples was measured and the relationship between 4β-OHC, *CYP3A5*3* genotype and tacrolimus exposure was examined. As another secondary exploratory study, prednisolone plasma concentrations were measured to explore the relationship between the above mentioned genetic polymorphisms and prednisolone exposure and its effect on tacrolimus dose.

A significantly lower tacrolimus exposure was observed in CYP3A5 expressers compared with CYP3A5 non-expressers for both formulations. In contrast to *CYP3A5*3* genotype, *ABCB1 3435C>T* gene had a minor influence on tacrolimus exposure irrespective of tacrolimus formulation. When combined, tacrolimus pharmacokinetics and dose requirements were significantly correlated with the combined-genotype grouping. The *CYP3A4*22 CT* genotype was associated with significantly greater tacrolimus exposure (AUC_{0-24}, C_{max}) compared with the *CYP3A4*22 CC* genotype. *POR*28 CT/TT* genotype was associated with significantly lower tacrolimus exposure compared with the *POR*28 CC* genotype in CYP3A5 non-expressing subjects.

Switching from immediate to prolonged release tacrolimus formulations in kidney transplant patients was associated with a significantly lower tacrolimus trough concentration (C_0), but had no influence on WPV. *CYP3A5* genotype had no impact on WPV. Plasma concentration of 4β-OHC was greater in CYP3A5 expressers. The 4β-OHC/C ratio was significantly correlated with tacrolimus exposure and dose requirement. Prednisolone exposure was not influenced by *CYP3A5*3, CYP3A4*22, ABCB1 3435C>T* or *POR*28* genotype.

Our results indicate that *CYP3A5*3*, *ABCB1 3435C>T* and *CYP3A4*22* polymorphisms are important determinants of tacrolimus disposition and may explain part of the clinically observed high between-individual variability in tacrolimus pharmacokinetics. *POR*28* is associated with tacrolimus dose requirement in CYP3A5 non-expressers. Thus, genotyping at these loci before renal transplantation may provide important information about the optimal initial dose of tacrolimus. Pharmacogenetic dosing strategies based on these genotypes are likely to be equally applicable to prescribing the once daily tacrolimus formulation, Advagraf®, as to twice daily formulations. Moreover, switching from immediate to prolonged release tacrolimus formulations had no influence on WPV. 4β-OHC/C ratio may be a useful biomarker for tacrolimus dosing in renal transplanted patients. Genotyping at *CYP3A5*3*, *CYP3A4*22*, *POR*28* and *ABCB1* 3435C>T loci is unlikely to allow individualization of prednisolone dose.

Publications

Abstracts

1. Elnahhas.T., Popoola.J., Ramkhelawon. R., MacPhee. I and Johnston. A. Tacrolimus intra-patient variability: The impact of conversion from immediate (Prograf®) to prolonged release (Advagraf®) tacrolimus formulations in stable renal transplant patients. BPS Winter Meeting, December 2012. London, UK.

2. T. El-Nahhas, J. Popoola, R. Ramkhelawon, A. Johnston, I. MacPhee. Intra-Patient Variability In Tacrolimus Trough Concentrations And Conversion Of Stable Renal Transplant Patients From Twice Daily (Prograf®) To Once Daily (Advagraf®). BTS Meeting, March 2013. Bournemouth, UK.

3. Toqa El-Nahhas, Michelle Moreton, Terry Lee, Denise McKeown, Joyce Popoola, Rajeshwar Ramkhelawon, Iain MacPhee, Atholl Johnston. *CYP3A4*22* as Pharmacogenomic Predictor of Tacrolimus Pharmacokinetics in Renal Transplant Recipients. BPS Winter Meeting, December 2013. London, UK.

4. Toqa El-Nahhas, Michelle Moreton, Terry Lee, Denise McKeown, Joyce Popoola, Rajeshwar Ramkhelawon, Iain MacPhee, Atholl Johnston. The Influence of P450 Oxidoreductase*28 Polymorphism on the Pharmacokinetics of Tacrolimus in Stable Renal Transplant Patients. BPS Winter Meeting, December 2013. London, UK.

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ABCB1	ATP Binding Cassette Subfamily B Member 1
Acc (%)	Accuracy
ADME	Drug Absorption, Distribution, Metabolism, and Elimination
amu	Atomic Mass Unit
ANOVA	Analysis of Variance
APC	Antigen-Presenting Cell
ATP	Adenosine Triphosphate
AUC	Area Under the Concentration versus Time Curve
AZA	Azathioprine
4β-OHC	4β-Hydroxycholesterol
4β-OHC/C	4β-Hydroxycholesterol/ Cholesterol
BP	Blood Pressure
C/D	Concentration/Dose
C_0	Trough Blood Concentrations
ºC	Degree Celsius
CaN	Calcineurin
CCD	Charge Coupled Device
CD4+	Helper T Lymphocytes
CD8+	Cytotoxic T Lymphocytes
CDK	Cyclin-Dependent Kinase
CE	Capillary Electrophoresis
CI	Confidence Interval
CKD	Chronic Kidney Disease
CKD-EPI	Chronic Kidney Disease Epidemiology Collaboration
CL/F	Apparent Total Clearance Of The Drug After Oral Administration
C_{max}	Maximum Concentration
C_{min}	Minimum Concentration
CNI	Calcineurin Inhibitor
CRF	Chronic Renal Failure
CsA	Ciclosporin
%CV	Coefficient Of Variation
CYP3A	Cytochrome P450, Family 3, Subfamily A
DBP	Diastolic Blood Pressure
ddNTPs	Dideoxynucleotide Triphosphates
dF/Dt	Rate Of Change In Fluorescence

dH_2O	Deionised Water
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide Triphosphates
EDTA	Ethylenediaminetetraacetic Acid
eGFR	Estimated Glomerular Filtration Rate
EMEA or EMA	European Medical Agency
ESI	Electrospray Ionization
ESRD	End Stage Renal Disease
EWP	Efficacy Working Party
FDA	Food And Drug Administration
FKBP	FK506-Binding Protein
FRET	Fluorescence Resonance Energy Transfer
g	Gram
GFR	Glomerular Filtration Rate
GIT	Gastrointestinal Tract
µg	Microgram
h	Hour
Hb	Haemoglobin
HLA	Human Leukocyte Antigens
HPLC	High Performance Liquid Chromatography
ICAM-1	Intercellular Adhesion Molecule-1
IL-2	Interleukin (IL) -2
IL-2R	Interleukin (IL) -2 Receptors
IQR	Interquartile Range
IS	Internal Standard
kDa	Kilo Daltons
kg	Kilogram
L	Litre
LC-MS/MS	High Performance Liquid Chromatography/Mass Spectrometry
LED	Light-Emitting Diode
LightCycler®	Gene-Expression and Melting Curve-Based Mutation Analysis System
LLOQ	Lower Limit Of Quantification
Log	Logarithm
LSS	Limiting Sampling Strategy
m	Month
M	Molar
m/z	Mass/Charge Ratio

mAbs	Monoclonal Antibodies
mbar	Megabar
MDR-1	Multi -Drug Resistance Gene-1
MEB	Medicines Evaluation Board in the Netherlands
mg	Milligram
MHC	Major Histocompatibility Complex
Millipore	Water Purification System
mL	Millilitre
mm	Millimetre
MMF	Mycophenolate Mofetil
MMS	Mycophenolate Sodium
MPA	Mycophenolic Acid
MPAG	7-O-Mpa-Glucuronide
MRM	Multiple Reaction Monitoring
mRNA	Messenger RNA
msec	Millisecond
MTBE	Methyl-Tert-Butyl Ether
Mut	Mutant (Variant) Allele
Mwt	Molecular Weight
NFAT	Nuclear Factor Of Activated T Lymphocytes
NF-ATc	Nuclear Factor of Activated T Lymphocytes (Inside the Cytoplasm)
NF-ATn	Nuclear Factor of Activated T Lymphocytes (Inside the Nucleus)
ng	Nanogram
NK	Natural Killer Cells
NODAT	New Onset Diabetes After Transplantation
NTI	Narrow Therapeutic Index
OD-Tac	Once-Daily Tacrolimus
PCR	Polymerase Chain Reaction
PCR-RFLP	Restriction Fragment Length Polymorphism Analysis of PCR-Amplified Fragments
P-gp	P-Glycoprotein
PK	Pharmacokinetics
pmoL	Picomole
pmp	Per Million Population
POR	P450 Oxidoreductase
PXR	Pregnane X Receptor
Q1	Parent Ion Scan
Q3	Product Ion Scan

QC	Quality Control
r	Correlation Coefficient
rpm	Revolutions Per Minute
RRT	Renal Replacement Therapy
RT-PCR	Real-time Polymerase Chain Reaction
SD	Standard Deviation
SAP	Shrimp Alkaline Phosphatase
SBP	Systolic Blood Pressure
SNP	Single-Nucleotide Polymorphism
Tac	Tacrolimus
Tac C_0	Tacrolimus Trough Blood Concentration
TBE buffer	Buffer Solution (Mixture of Tris Base, Boric Acid and EDTA)
TCR	T-Cell Receptor
TD-Tac	Twice-Daily Tacrolimus
TDM	Therapeutic Drug Monitoring
TI	Therapeutic Index
Tm	Melting Temperature
t_{max}	Time to Achieve Maximum Concentration
TOR	Target of Rapamycin Protein
ULOQ	Upper Limit of Quantitation
UPLC	Ultra-Performance Liquid Chromatography
UV	Ultraviolet
V	Volt
w	Week
WPV	Within-Patient Variability
Wt	Homozygous Wild Type

Chapter 1. General Introduction and Thesis Summary

The immunosuppressive drug tacrolimus is widely used to prevent the rejection of renal transplants. Tacrolimus was initially available as a preparation requiring twice daily administration: Prograf®. A modified-release formulation of tacrolimus (once-daily; Advagraf®) has been developed to provide more consistent exposure and facilitate better adherence. Tacrolimus has a narrow therapeutic index with wide variation between individuals in the blood concentration achieved by a given dose. To some extent, this is influenced by genetic factors. The influence of the *CYP3A5*3* and *ABCB1 3435* genotypes on the pharmacokinetics of immediate release tacrolimus; Prograf® is well-defined. However, the influence is unclear for prolonged release tacrolimus; Advagraf®. High within-patient tacrolimus trough concentration variability is a risk factor for long term chronic allograft nephropathy and graft loss. Therapeutic drug monitoring is used routinely to direct tacrolimus dosing within a defined therapeutic range of whole blood concentrations. Maintenance of the target blood concentration of immunosuppressive drugs is one of the main factors determining transplant function. Finding the optimal dose that gives the right balance between efficacy and toxicity remains the challenge for immunosuppressive drugs, especially during the period immediately after transplantation. Therefore, the identification of parameters predictive of the optimal initial tacrolimus dose has the potential to improve clinical outcomes. In **chapter 2** of this thesis, a description of renal failure stages, epidemiology and risk factors leading to its development are included. An overview of the role of the immune system in transplant rejection and the importance of use and monitoring immunosuppressive drugs is described. Following this general background, the concept of bioequivalence and genotyping studies is given. In **chapter 3,** an overview of tacrolimus and its pharmacokinetics is covered. Tacrolimus exposure has been related to clinical outcome and therapeutic drug monitoring is often applied in clinical practice to guide personalized dosing. Tacrolimus has a large between- and within-patient variability in its exposure. Factors considered to cause this variability are outlined in this chapter. In **chapter 4 and 5** genetic markers that are related to between-patient variability in

tacrolimus exposure are determined. The influence of *CYP3A5*3*, *CYP3A4*22*, *POR*28* and *ABCB1*3435C>T SNPs on the pharmacokinetics of immediate release tacrolimus; Prograf® or Adoport® and prolonged release; Advagraf® was carried out within individual patients. Moreover, in **chapter 4**, genes encoding for CYP3A5 (*CYP3A5*3*), *ABCB1* gene 3435C>T functional polymorphism and their combination were analysed in relation to tacrolimus pharmacokinetics in 64 adult renal transplant patients for both immediate and prolonged release formulations. In this study a strong association of *CYP3A5*3* genotype (rs776746) with tacrolimus exposure for Advagraf® as well as for Prograf®/Adoport® was observed. CYP3A5 expression had a major influence and *ABCB1 3435* genotype had a minor influence on tacrolimus exposure for both formulations. Tacrolimus exposure and dose requirement were significantly associated with the combined-genotype grouping. The daily doses for CYP3A5 expressers/Pg-p high-expressers and CYP3A5 expressers/Pg-p intermediate- and poor-expressers were 2.6- and.1.8-fold higher than CYP3A5 non-expressers/Pg-p intermediate- and poor-expressers, respectively. In **chapter 5**, a comprehensive pharmacogenetic analysis was performed for recently identified genes encoding for CYP3A4 (*CYP3A4*22*) and for *POR*28* in relation to tacrolimus exposure for both immediate and prolonged release formulations. In this study, novel methods were developed for genotyping *CYP3A4*22* and *POR*28* SNP using real time PCR; LightCycler® based technique. In this study only *CYP3A4*22* SNP was significantly associated with tacrolimus exposure. *POR*28* genotype was only associated with tacrolimus exposure in CYP3A5 non-expressers. The influence of the *CYP3A4*22* and *POR*28* genotype on tacrolimus exposure was the same for the prolonged release preparation Advagraf® as for the immediate release preparation, Prograf® or Adoport®. In **chapter 6**, the study was conducted with the intention of exploring the relationship between 4β-hydroxycholesterol, a plasma biomarker of cytochrome P4503A activity, and *CYP3A5*3* genotype and tacrolimus exposure. In this study *CYP3A5*3* allele was found to have a significant effect on the plasma 4β-OHC concentration in stable kidney transplant recipients. The 4β-OHC/C ratio was significantly correlated with *CYP3A5*3* and tacrolimus dose requirement. **Chapter 7** focuses on tacrolimus within-patient variability in 100 stable kidney transplant recipients. Only few studies have focused on

comparing within-patient variability of tacrolimus trough concentration after conversion from immediate to prolonged tacrolimus formulations in stable renal transplant recipients. In this study, switching from immediate to prolonged release tacrolimus formulations had no influence on within-patient variability. This study also investigated the correlation between tacrolimus within-patient variability and *CYP3A5*3* genotype in stable kidney transplant recipients for twice daily tacrolimus (Prograf® or Adoport®) and once daily tacrolimus, Advagraf®. Very few studies have focused on the relation between within-patient variability and genetic polymorphisms, particularly *CYP3A5*3* polymorphism in twice daily tacrolimus formulation. In this study, *CYP3A5*3* genotype was not related to the within-patient variability of tacrolimus trough concentration in both immediate and prolonged tacrolimus formulations. In **chapter 8** a new simple and rapid bioanalytical method for measurement of prednisolone and its metabolite in plasma using LC-MS/MS was developed and validated. This method provides the opportunity to assess prednisolone and prednisone pharmacokinetics in a large cohort of renal transplant patients. In this study the relationship between the *CYP3A5*3*, *ABCB1*, *CYP3A4*22* and *POR*28* SNPs and prednisolone exposure was investigated. None of the investigated SNPs were associated with prednisolone pharmacokinetics. In **chapter 9,** the results presented in the studies described earlier were analysed with reference to findings of previous research in the same area of interest or using similar techniques. Discussion of the genotype–phenotype associations with Advagraf® daily dose requirements was then made. The discussion focuses on evaluating the effects of conversion from immediate to prolonged released tacrolimus formulation from three perspectives: 1) Genetic influence on tacrolimus pharmacokinetics, 2) Within-patient variability differences, and 3) Suggestions for a pharmacogenetic strategy to guide individualisation of twice daily tacrolimus and Advagraf® dose. **Chapter 10** will describe briefly the potential studies that will be performed in the future.

Chapter 2. Background

2.1 Renal Failure and Immunosuppression Therapy

2.1.1 Renal Failure

Chronic kidney disease (CKD) is a public health problem with a growing prevalence worldwide. Glomerular filtration rate (GFR) is considered the best general guide of deteriorating renal function. Low GFR is associated with an increased risk of kidney failure requiring dialysis and complications such as cardiovascular disease, hypertension, anaemia, and other metabolic complications (Himmelfarb and Sayegh, 2010). Renal failure is defined as the loss of renal function leading to a fall of GFR below 90 mL/min and to an accumulation of creatinine, urea and other nitrogenous wastes. Based on the level of estimated GFR normalized to body surface area, CKD has been classified into five stages (**Table 1**), with stage 1 being the mildest and stage 5 the most severe (NHS, 2010).

Table 1: Stages of Chronic Kidney Disease.

Stage	Description	GFR* mL/min/1.73m^2
1	Slight kidney damage with normal or increased GFR	≥ 90
2	Mild decrease in kidney function	60-89
3a	Moderate decrease in kidney function	45–59
3b		30–44
4	Severe decrease in kidney function	15-29
5	Kidney failure	< 15

*****All GFR values are normalized to an average surface area (size) of 1.73m^2

2.1.1.1 Epidemiology of Chronic Kidney Disease

The number of patients with CKD is increasing worldwide and it reflects the increase in end stage renal disease (ESRD) patients treated by renal replacement therapy (RRT) including dialysis or transplantation. In the UK, in 2013 the annual incidence of ESRD

was 109 new patients per million of population with a stable incidence in England over the last 8 years (Gilg et al., 2015). However, this figure remains below the European average (about 135 per million) and that of the United States (336 per million) (Hamer and El Nahas, 2006). At the end of 2013, the number of adult patients in UK receiving RRT was 56,940, giving a prevalence of 888 per million population (pmp). The growth in the prevalent UK RRT population from the previous year was 4.0%. The annual growth rate has been fairly consistent over the last 10–15 years (Rao et al., 2015).

2.1.1.2 Risk Factors for Chronic Kidney Disease

The common aetiologies for chronic renal failure include arteriopathic renal disease, hypertension, glomerulonephritis, diabetes mellitus, infective, obstructive and reflux nephropathies, hereditary kidney disease, hypercalcaemia, connective tissue diseases and myeloma (Brown et al., 2009, Arora, 2013). Several factors play a role in the rising incidence of ESRD.

2.1.1.2.1 Advancing Age

Chronic kidney disease is a major health problem, mainly a disease of elderly people. The annual incidence rate of CKD in the UK rose from 60 per million among patients aged 20-49 to 282 per million among those in their 60s and 588 among those in their 80s (Feest et al., 1990). The prevalence is less than 1% in people aged 18 to 25, this increases to more than 40% in the 85 and over age population (NHS, 2010). A growing incidence of ESRD in elderly patients has also been observed in other countries. The overall annual incidence of chronic renal failure (CRF) in France rose from 64/million in patients under 40 years old up to 356/million in patients over 75 (Jungers et al., 1996). The incidence rate of ESRD per year in the United States has been generally stable for the past two decades among people aged 20-44 years old. On the other hand, the number of patients aged greater than 45 years and above had been increasing for many years, with particularly dramatic increases for age 65 and above. The prevalence of ESRD steeply rises among patients aged greater than 45 patients years (USRDS, 2014). In the UK, the incidence rates for all age groups have plateaued in the last eight years. People up to 44 years old have the lowest incidence rate followed by the 45–64

age group. However, over 65 years old population have the highest incidence rate (Gilg et al., 2015). The considerable increase in the incidence of CRF with advanced age is mainly due to the presence of additional factors and / or complications that might be linked with a reduction in the number or the function of nephrons. The high incidence of chronic renal failure in elderly patients is probably due to the high prevalence of renal vascular disease in this age group (Jungers et al., 1996).

2.1.1.2.2 Sex

Although, the increase in the incidence of CRF with age was similar in both males and females, the incidence of CRF was strikingly higher in male as compared to female patients. The annual incidence was twice as high in males compared to females up to 75 years and three times as high in patients above 75 years (Jungers et al., 1996). End stage renal disease prevalence rate in males exceeded that of females for all age groups, reaching the highest point in the age group 75–79 years at 3,010 pmp in men and for women at 1,560 pmp (Rao et al., 2015). Typically, renal replacement populations are around 60% male and 40% female.

2.1.1.2.3 Ethnicity

Some ethnic groups have higher rates of CKD than others. For example, in the UK and USA, the incidence of RRT in African-Caribbean and South Asian populations in any age group is 3 to 5 times higher than in Caucasians. In addition, in the USA, Black Americans have at least 4 to 5 times higher RRT rate than whites. The annual incidence is 256/1 000 000 among white people compared with 982/1 000 000 in African-Americans (Hamer and El Nahas, 2006, El Nahas and Bello, 2005). Moreover, in the year 2012, the prevalence rate per million in the USA remains much higher in blacks/African Americans than in other ethnic groups, at approximately 2.5-fold higher as compared to Asians, and 4-fold higher compared to Whites. The prevalence rate was 5,671 per million in black/African Americans, 2,272 per million in Asians, and 1,432 per million in Whites (USRDS, 2014).

2.1.1.2.4 Diabetes

Impaired kidney function is a common and serious complication of diabetes mellitus. Estimates indicate that 25–40% of patients with Type 1 or Type 2 diabetes develop diabetic nephropathy within 20–25 years of disease onset (New et al., 2007). Several studies have highlighted that diabetes is strongly associated with the increased risk for CKD. The prevalence of a reduced GFR 60 ml/min per 1.73 m^2 was threefold higher in those with diabetes mellitus compared with those without (Chadban et al., 2003). A linear relationship was reported between hyperglycemia and both the development and progression of diabetic nephropathy in type 1 and type 2 diabetes patients (Hall, 2006). In 2013, the UK Renal Registry found that diabetes accounted for 15.9% of renal disease in prevalent patients on RRT, and it was more common among patients aged 65 than among younger patients (Rao et al., 2015). It has been reported that Type 1 and Type 2 diabetes patients had more than twice the risk of ESRD compared with non-diabetic patients and the highest risks of moderate-severe CKD occurred with Type 1 diabetes (Hippisley-Cox and Coupland, 2010). Another study demonstrated that diabetic patients had 1.5 fold higher chances for developing CKD than individuals without diabetes after adjustment for age (Hallan et al., 2006).

2.1.1.2.5 Hypertension

High blood pressure (BP) is strongly associated with CKD in men and women (Haroun et al., 2003). Several studies have shown that hypertension is a risk factor for CKD. The prevalence of proteinuria and reduced GFR <60 ml/min per 1.73 m^2 were fivefold higher in hypertensive participants compared with those without hypertension (Chadban et al., 2003). Hallan et al. (2006) found that individuals with hypertension had five-times more prevalence of CKD than those with normal BP and after adjustment for age the risk ratio was 1.5. A recent UK study showed that hypertensive patients had more than double the risk of ESRD compared with patients without hypertension (Hippisley-Cox and Coupland, 2010). In the USA, the incidence rates of ESRD due to hypertension in all age groups are dramatically greater in Blacks/African Americans compared with the other ethnic groups. Incidence rates in Blacks/African Americans are over ten-fold

higher than in Whites in the young population and 2.5 times more than whites in the age group 70 and over (USRDS, 2014).

2.1.2 Renal Replacement Therapy

In chronic renal failure, the loss of renal function is slow and progressive and in most cases the damage is irreversible. Eventually, the kidneys cease to function. This condition is referred to as end-stage renal failure (ESRF) (Abuelo, 1995). In this case, renal replacement therapy is required. Treatment methods cannot cure the renal failure. However, it is possible to replace renal function through the use of alternative methods that include dialysis and kidney transplantation (Stein and Wild, 2002). The process of dialysis bypasses the kidneys, in order to remove metabolic wastes and foreign compounds artificially. This maintains fluid and electrolyte balance within acceptable limits and removes toxins. However, the plasma cleansing technique does not make up for the kidneys' reduced ability to produce hormones such as erythropoietin and renin and to activate vitamin D (Sherwood, 2008). Additionally, there is a considerable time commitment for patients undergoing dialysis. Consequently, kidney transplantation is the replacement therapy of choice for end-stage renal disease. Quality of life is greatly enhanced by renal transplantation and survival is probably better than that for patients on dialysis (Wolfe et al., 1999). It is also the most cost-effective mode of renal replacement therapy (Singh and Bhandari, 2004).

Early graft survival has improved markedly in recent years, but long-term survival remains a significant challenge (Chandraker et al., 2011). The risk of acute renal allograft rejection is greatest during the first three months following transplantation. Therefore, developing protocols that optimise immunosuppression during the high immunologic risk period has the potential to limit damage to the graft that has an adverse influence on long term graft function (Velia, 2005).

2.1.3 The Role of the Immune System in Transplantation

The immune system defends the body from harmful substances by distinguishing and reacting to antigens that include toxins, viruses, bacteria, transferred blood cells, and

transplanted organs (Male et al., 2006). A transplanted kidney is recognized by the immune system as an antigen and this triggers the immune response and the subsequent rejection of the organ. Based on the speed and specificity of the response, the immune system is divided into two main parts; innate and the adaptive immunity (Parkin and Cohen, 2001).

2.1.3.1 Innate Immune Response

It is a series of nonspecific defence mechanisms to protect the body against infection. These defence mechanisms are natural, do not require exposure to a specific antigen and work immediately or within few hours after exposure to an inflammatory stimulus. The components of the innate immune system include anatomical barriers, secretory molecules and cellular components. The mechanical anatomical barriers comprise the mechanical, chemical and biological elements. Mechanical barriers are well-known as skin, mucous membrane and ciliated epithelial cells while chemical barriers include the low pH of sweat and gastric secretions, lysozymes, phospholipase, and interferons and biological barriers contain the normal microbial flora of the skin and in the gastrointestinal tract. Innate immunity also has some cellular barriers, which are the primary defence line of the immune system. The cells can be divided into granulocytes (neutrophils, basophils and eosinophils), monocytes which develop into macrophages and lymphocytes (B, T and natural killer (NK) cells), see **Figure 1**. Monocytes and macrophages attract, engulf, and ingest foreign bodies. Basophils release histamine and they are important in defence against parasites. Once activated, eosinophils produce a range of highly toxic proteins and free radicals that are extremely efficient in destroying bacteria and parasites. Natural killer cells and lymphokine activated killer cells can non-specifically kill virus infected and tumour cells. The complement system is the major humoral non-specific defence mechanism. Upon activation, complement can lead to increased vascular permeability, recruitment of phagocytic cells, and lysis and opsonization of bacteria. Some coagulation system products can participate in the non-specific defences, as they have the ability to increase vascular permeability and act as chemotactic agents for phagocytic cells (Mayer, 2011, Williams et al., 2012).

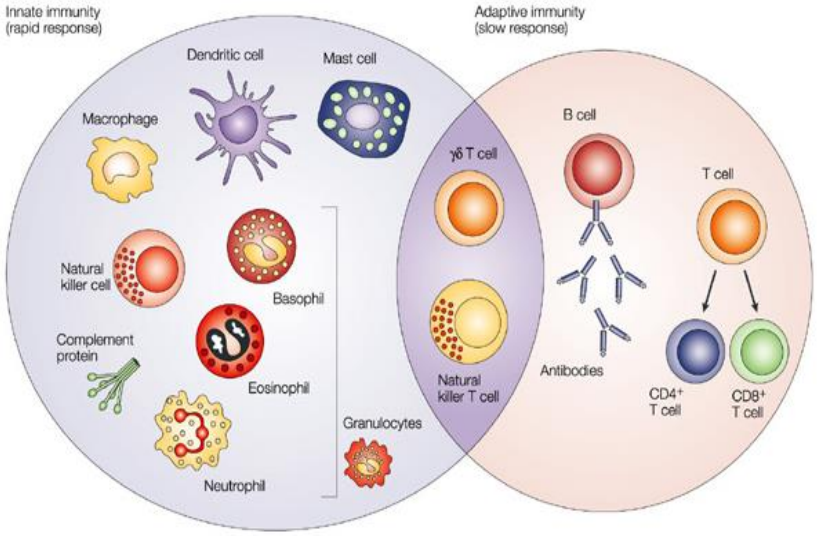


Figure 1: The Main Components of the Innate and Adaptive Immune Systems (Dranoff, 2004). The innate immune response is the short-term reaction. It consists of granulocytes (basophils, eosinophils and neutrophils), mast cells, macrophages, dendritic cells, complement proteins and natural killer cells. The adaptive immune response is the longer-term reaction. It consists of antibodies, B lymphocytes, and helper (CD4+) and cytotoxic (CD8+) T lymphocytes. Natural killer T lymphocytes and gammadelta T lymphocytes are cytotoxic lymphocytes that straddle the interface of innate and adaptive immunity.

2.1.3.2 Adaptive Immune Systems

The adaptive immunity is an antigen-specific immune reaction. It is more complex than innate immunity. The antigen first must be processed and identified. Once an antigen has been recognized, the adaptive immune system stimulates potent mechanisms for neutralizing or killing pathogens. The adaptive immune system components are normally silent and require activation with the innate immune response promoting activation initially. In transplantation the surgery promotes an inflammatory response that is an important factor in triggering the adaptive immune response to cause transplant rejection. The response is slower compared with the innate immune response but it is much more potent. There are two categories of adaptive immune responses: humoral immunity, which mediated by B-lymphocytes producing antibodies, and cell-mediated immunity which mediated by T lymphocytes, see **Figure 1** (Alberts et al., 2002). The acquired immune system also plays a fundamental role in the rejection of

transplanted organs and destroying the graft. After maturation, mature T lymphocytes move through the circulation to lymph nodes, mucosa-associated lymphoid tissue or the spleen. B lymphocytes on the other hand, pass through the circulation to various sites throughout the body.

Once activated with a foreign antigen, B lymphocytes develop to mature antibody secreting cells called plasma cells and start producing antibodies, which contribute to the immunity in three different mechanisms: neutralisation via binding to antigens and preventing them from invading cells and directly neutralize them; opsonisation stimulates pathogens elimination by phagocytic cells and complement fixation where antigen-antibody complex triggers cascade complement activation and consequently antigen lysis. Antibodies are also known as immunoglobulins and they are divided into 5 major types IgA, IgE, IgG, IgM and IgD. B lymphocytes are important in the fight against pyogenic bacteria and facilitate hyper acute rejection and can destroy a graft by producing antibodies (Atluri et al., 2006).

Unlike B lymphocytes, T lymphocytes do not recognize antigens directly. Instead, there are receptors located in their surfaces that identify peptide fragments of antigens associated with major histocompatibility complex (MHC) molecules on the surfaces of infected or cancerous cells. The major histocompatibility complex is a set of genes that encodes a group of proteins that are located on the cell surface. There are several types of T lymphocytes: T helper lymphocytes (Th or CD4+), cytotoxic T (Tc or CD8+) lymphocytes, memory T (Tm) lymphocytes and suppressor T (Ts) or regulatory T (Treg) lymphocytes. Cytotoxic T lymphocytes (Tc) and memory T lymphocytes (Tm) lymphocytes are the effector cells, whereas helper lymphocytes (Th) and suppressor lymphocytes (Ts) are regulatory cells. T lymphocytes contribute to the immune defences in two major ways: some direct and regulate immune responses, whereas others directly attack infected or cancerous cells. Th lymphocytes are divided into Th1 and Th2, where Th1 produces cytokines and lymphokines (IL-2, IL-12 and gamma-interferon) that activate macrophages and participate in the production of cytotoxic lymphocytes. In contrast cytokines produced by Th2 lymphocytes help to activate B lymphocytes, resulting in antibody production. Cytotoxic T (Tc) lymphocytes directly

attack and destroy infected cells. Two main proteins, granzymes and perforin are stored in cytoplasmic vacuoles within the resting Tc lymphocytes. When a Tc lymphocyte encounters an infected cell presenting on its surface MHC-bound antigenic peptide, it is stimulated to release perforin into the intracellular space between the Tc and the target cell, which form holes in the surface membrane of the target cell and this results not only in direct cell damage, but also provide means for granzymes to enter the cell. Granzymes induce target cells to undergo apoptosis. Regulatory T (Treg) lymphocytes inhibit immune response after invading organisms are destroyed by release chemicals that decrease B- and T- lymphocytes activity and division. Memory T lymphocytes remain in the body for several years. Once they have encountered an antigen and cloned to remember it, they are responsible for defence on repeated exposure (Atluri et al., 2006).

The antigen can only be recognized by T lymphocytes if one of the body's own MHC molecules carries it on the cell surface. In humans, MHC antigens are called human leukocyte antigens, or HLA and the genes are located in the short arm of chromosome 6. T lymphocytes recognize MHC molecules when they differentiate between self and non-self. A self-MHC molecule provides an identifiable scaffolding to display a foreign antigen to the T lymphocytes. MHC Class I proteins are found on all nucleated cell surfaces and they present antigens to cytotoxic T lymphocytes (Tc). In the resting state MHC Class II proteins are found only on B lymphocytes, macrophages, and other antigen presenting cells. Inflammatory cytokines promote the expression of MHC class II molecules by other tissues. There is a wide diversity between individuals in the protein sequence of the MHC molecules expressed on the cell surface which has probably minimised the risks of microbial evolution to evade the immune response. A consequence of this is that MHC moleules on transplanted organs are recognised as being 'foreign' triggering an immune response against the organ (Penn, 2002). Consequently, organ donors should ideally have the closest MHC match with recipients to minimise the risk of graft rejection.

2.1.3.3 Mechanism of Alloimmune Responses

The transplanted organ is a permanent source of antigens and it can induce rejection at any time following transplantation. The immune system establishes effective mechanisms to fight the transplanted organ, which are recognized by the immune system as a foreign agent and is termed transplant rejection. Rejection is an acquired immune response via cellular immunity (mediated by T-lymphocytes) and this involves the production of cytotoxic T-lymphocytes, activated macrophages, activated NK cells, and cytokines in response to the foreign antigen inducing apoptosis of target cells. It also involves humoral immunity, which includes the production of antibody molecules in response to the transplanted organ and is mediated by B-lymphocytes though the action is joined by components of innate immune response (phagocytes and soluble immune proteins). The degree to which allografts undergo rejection depends partly on the degree of similarity or histocompatibility between the donor and the recipient (Malhotra et al., 2013).

Following organ transplantation, a series of immunological reactions occurs, which comprise inflammation, immunity, and tissue repair, as well as structural reinforcement of damaged cells. Inflammation occurs at the site of transplantation and is mediated by macrophages, T lymphocytes and pro-inflammatory mediators (eg, IL-2). Complement activation results in the elaboration of bioactive intermediates such as C3a and C5a. Once the immune system destroys the antigens, macrophages, endothelial cells, smooth muscle cells, and fibroblasts begin to promote tissue repair and reinforcement. However, rejection takes place when a strong inflammatory response develops or when tissue repair and remodeling fail (Prescilla, 2013).

Rejection is classified into 3 broad categories according to the timing of the rejection. The first category is termed hyperacute rejection, which may develop within minutes to hours of graft implantation because of the presence of pre-formed anti-donor antibody. The antigen-antibody complexes activate the complement system, causing massive thrombosis in the capillaries, resulting in ischemic damage of the graft. However, this condition is rare nowadays as a routine pre-transplant anti-donor antibody screen is

introduced. The second type of rejection is acute rejection which most commonly occurs during the first 3 months after transplantation. The reaction is unusual before 1 week after transplantation, the typical time lag for development of a primary immune response. Once T lymphocytes encounter the graft alloantigens, they stimulate the release of cytokines and consequently results in tissue distortion, vascular insufficiency, and cell damage. However, this reaction can be prevented by the use of immunosuppressant drugs, allowing tissue repair to progress. The third rejection category is termed chronic rejection, where pathologic tissue remodelling and reinforcement takes place. Blood flow is reduced, which contribute to regional tissue ischaemia, fibrosis, and cell death. This type of rejection cannot be cured as there is no treatment available (Prescilla, 2013). There is increasing recognition of the importance of alloantibody in chronic allograft rejection (Sellares et al., 2012).

2.1.4 Immunosuppressive Therapy

Rejection is the main barrier to successful transplantation. Therefore, the use of immunosuppressive drugs is very important in order to suppress the immune system and to prevent rejection of the transplanted organ. Large doses are used immediately after the transplantation and reduced over time as the risk of acute rejection reduces. Immunosuppressive drugs have many unpleasant adverse effects both due to inhibition of protective immune responses leading to increased risk of infection and malignancy and a number of drug-specific complications, including nephrotoxicity and weight gain. The use of combination immunosuppressive therapy has developed over a number of years in order to maximise synergistic effect and to minimize side effects and toxicity (Meier-Kriesche et al., 2011). In current practice, most patients are given an induction antibody that either depletes or blocks the activation of lymphocytes at the time of transplantation and maintenance immunosuppression that is generally orally administered small molecule drugs. The mechanisms of action of the most commonly used immunosuppressive drugs in organ transplantation are shown in **Figure 2** and summarized in **Table 2**.

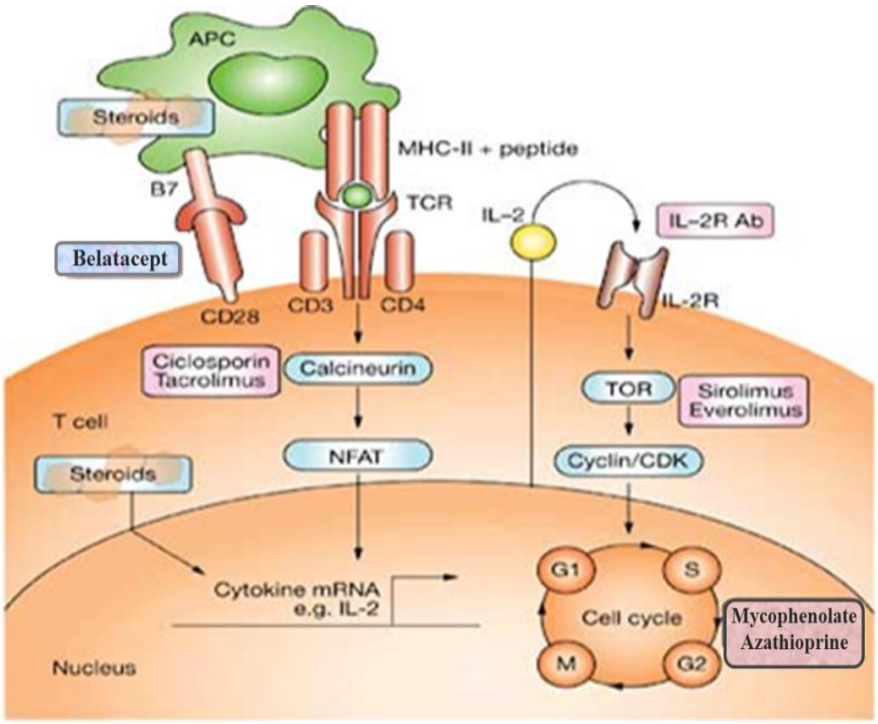


Figure 2: Mechanisms of Action of Immunosuppressive Drugs (modified from (Kobashigawa and Patel, 2006).

Immunosuppressive drugs inhibit a number of steps in the mechanisms leading to T-lymphocytes proliferation. Steroids inhibit antigen presentation, cytokine production. Ciclosporin and tacrolimus inhibit calcineurin activation and interleukin-2 production that resulted from donor peptide presentation by antigen-presenting cells in conjunction with the major histocompatibility complex class II and B7 complex. Interleukin-2 production leads to cell proliferation by a pathway involving target of rapamycin and cyclin/cyclin-dependent kinase that known for their role in regulating the cell cycle. IL-2R Ab blocks Interleukin (IL)-2 receptors and inhibits IL-2-induced T- lymphocytes proliferation. Sirolimus and everolimus inhibit target of rapamycin and interleukin-2-driven T-lymphocytes proliferation. Mycophenolate and Azathioprine inhibit synthesis of the purine building blocks of DNA and prevent T lymphocytes proliferation. Belatacept inhibits the CD28-CD80/CD86 (B7) interaction resulting in the blockade of T-lymphocyte activation.

APC, antigen-presenting cell; CDK, cyclin-dependent kinase; IL-2, interleukin-2; IL-2R, interleukin-2 receptor; IL-2R Ab, interleukin-2-receptor antibody; MHC, major histocompatibility complex; MMF, mycophenolate mofetil; mRNA, messenger RNA; NFAT, nuclear factor of activated T lymphocytes; TCR, T-cell receptor; TOR, target of rapamycin protein. G1 (first growth phase), S (synthesis of DNA), G2 (second growth phase) and M (cell division) represent the phases of the cell cycle.

Table 2: Classification and Mechanisms of Action of Drugs Commonly Used in Transplantation.

Drug Class/ Drug	Mechanism of Action
Corticosteroid Prednisone/Prednisolone	Corticosteroids inhibit antigen presentation and cytokine production. They are potent inhibitors of tissue damaging inflammation.
Calcineurin inhibitor (CNI)	CNIs block the intracellular T- lymphocytes signals responsible for the production of cytokines CNIs block the intracellular T-lymphocytes signals responsible for the production of cytokines (in particular IL-2, interferon-gamma and tumour necrosis factor alpha).
Ciclosporin (CsA)	CsA binds to cyclophilin; complex inhibits calcineurin phosphatase and T- lymphocytes activation.
Tacrolimus (TAC)	TAC binds to FKBP12; complex inhibits calcineurin phosphatase and T- lymphocytes activation.
Antimetabolite	Antimetabolites inhibit synthesis of the purine building blocks of DNA, namely guanine and adenine.
Azathioprine (AZA)	AZA is converted to 6-mercaptopurine and then to 6-thioinosine monophosphate – a precursor of thioguanine nucleotides that inhibit purine synthesis and interfere with DNA and RNA replication.
Mycophenolate (MMF & MPS)	Mycophenolic acid (mycophenolate) inhibits synthesis of guanosine monophosphate nucleotides and blocks purine synthesis, preventing proliferation of T and B lymphocytes.
Mammalian target of rapamycin (mTOR)	mTOR inhibitors act by blocking the serine-threonine kinase mTOR.
Sirolimus (SRL)	SRL binds to FKBP12; complex inhibits target of rapamycin and interleukin-2-driven T- lymphocytes proliferation.
Everolimus (ERL)	ERL binds to FKBP12; complex inhibits target of rapamycin and interleukin-2-driven T- lymphocytes proliferation.
Interleukin (IL)-2 receptor antagonist	The anti-IL-2R mAbs are specific for the alpha subunit (CD25) of IL-2Rs on activated T lymphocytes. They saturate IL-2Rs and, thus, are competitive antagonists of IL-2-induced T- lymphocytes proliferation.
Basiliximab	Basiliximab binds to and blocks the IL-2R alpha chain (CD25 antigen) on activated T lymphocytes, inhibiting IL-2-induced T-cell activation.

Lytic monoclonal antibodies	Monoclonal antibodies are monospecific antibodies made by one type of immune cell that are all clones of a unique parent cell that specifically bind to a target cell.
Alemtuzumab	Alemtuzumab binds to CD52, a protein present on the surface of mature lymphocytes, leading to lymphocyte lysis via complement activation and antibody dependent cellular cytotoxicity.
Rituximab	Rituximab binds to CD20, a protein present on the surface of immune system B lymphocytes, the combined effect results in the elimination of B lymphocytes.
Polyclonal antibody	Polyclonal antibody preparations are purified immunoglobulin preparations derived from animals after immunization with human thymocytes. The polyclonal antibodies currently available are all ATGs obtained by immunization of horses (equine antithymocyte globulin (eATG) or rabbits (rATG) that blocks T- lymphocyte membrane proteins, causing altered function, lysis, and prolonged T- lymphocyte depletion.
Belatacept	Belatacept inhibits the CD28-B7 interaction resulting in the blockade of T-cell activation.

Immunosuppressive drug therapy following transplantation is divided into three main phases: induction phase, which is used immediately following transplantation to provide an intense immunosuppression, Maintenance phase where drugs are used for long term prophylaxis of immunosuppression as well as rejection reversal or rescue phase where drugs are used for treatment of an established problem (Fogel and Greenberg, 2015). The patients are usually maintained using a triple therapy consisting of calcinurin inhibitor, low dose prednisolone and mycophenolate or azathioprine. Moreover, the prednisolone dose is gradually reduced over time and completely withdrawn in some patients (Jorga and Johnston, 2005).

2.1.4.1 Corticosteroids

Corticosteroids interrupt multiple steps in immune activation because of the ubiquitous expression of corticosteroid receptors. Prednisolone is a synthetic adrenocortical steroid derivative with predominantly glucocorticoid properties possessing anti-inflammatory and immunosuppressive action (Adcock and Ito, 2000). Its anti-inflammatory properties are apparent through preventing or suppressing the tissue response to the inflammatory process. Complications of corticosteroid therapy are numerous and virtually any organ system in the body may be affected. Short-term or acute adverse effects, which usually occur with initiation of therapy, include central nervous system effects, psychosis, pseudotumor cerebri, impaired glucose tolerance, and retention of sodium and fluid. This may exacerbate congestive heart disease and may aggravate preexisting diabetes mellitus. Long-term adverse effects can lead to the development of iatrogenic Cushing syndrome with truncal obesity. It also increases the susceptability to infections of certain species including Aspergillus species, Pneumocystis jiroveci, and Herpes virus species (Stanbury and Graham, 1998). Prednisolone is readily absorbed from the gastrointestinal tract. It is extensively bound to plasma proteins and has a usual plasma half-life of 2 to 3 hours. Prednisolone is excreted in the urine as free and conjugated metabolites together with a considerable proportion of unchanged prednisolone (Martindale and Reynolds, 1996). Prednisolone is metabolised by enzymes in the CYP3A family (CYP3A4/CYP3A5) and it is a substrate of the efflux transporter P-glycoprotein. Prednisolone is a CYP3A and P-glycoprotein inducer with consequent impact on the metabolism of other drugs (Miura et al., 2008).

2.1.4.2 Calcineurin Inhibitor (CNI)

Calcineurin inhibitors block the intracellular T-cell signals responsible for the production of cytokines. This group of drugs includes ciclosporin (CsA) and tacrolimus (TAC).

2.1.4.2.1 Ciclosporin (CsA)

Ciclosporin CsA is a cyclic nonribosomal peptide of 11 amino acids and contains a single D-amino acid, which are rarely encountered in nature. It was initially isolated from a fungus called *Tolypocladium inflatum* (Beauveria nivea). It is the first calcineurin inhibitor used in transplantation and was approved by the US Food and Drug Administration in 1983. It remains the primary agent in immunosuppressive regimens in many transplant programmes worldwide. Ciclosporin acts through the production of a complex with cyclophilin that inhibits calcineurin phosphatase activity. Consequently, CsA inhibits translocation of the nuclear factor of activated T lymphocytes (NFAT) family of transcription factors from the cytoplasm to the nucleus of activated T lymphocytes. It also inhibits lymphokine production and interleukin release (in particular IL-2, interferon gamma and tumour necrosis factor alpha) and, therefore, inhibits T cell activation and proliferation (Finkel et al., 2009). The common adverse effects of CsA include nephrotoxicity, hypercholesterolaemia, systemic hypertension, gingival hyperplasia, hirsutism hypertrichosis, and neurotoxicity. Impairment of glucose tolerance is an important complication of the CNIs resulting in New Onset Diabetes After Transplantation (NODAT). In addition, it increases vulnerability to opportunistic fungal and viral infections and displays a possible role in promoting cancer progression and tumour cell invasion as well as metastasis (Prescilla, 2013).

Ciclosporin is available for intravenous or oral administration. It is provided as soft gelatin capsules and oral solutions. The absorption of the original soft gelatin capsule (SANDIMMUNE®) is slow, with 20% to 50% bioavailability. However, the modified microemulsion formulation (NEORAL®) has a slightly increased bioavailability compared to SANDIMMUNE®. After oral administration, Ciclosporin absorption is

incomplete and varies among the individual patient and the formulation used. Ciclosporin reaches its peak blood concentration within 1.5 to 2 hours and its absorption is greatly decreased by food. Ciclosporin is extensively metabolized by CYP3A in the liver. It is mainly excreted through the bile into the faeces and only 0.1% of ciclosporin is excreted unchanged in the urine (Goodman et al., 2006).

2.1.4.2.2 Tacrolimus (TAC)

Tacrolimus is the most widely used calcineurin inhibitor in renal transplant recipients in the United Kingdom. It is a macrolide lactone antibiotic produced by Streptomyces tsukubaensis discovered in 1984 from the fermentation broth of a Japanese soil sample. Tacrolimus was first approved by the Food and Drug Administration in 1994 for use in liver transplantation and later also for use in kidney transplantation. Tacrolimus is considered as one of the most potent immunosuppressive agents used in managing rejection following renal transplantation and belongs, together with CsA to a unique class of immunosuppressive natural products known as Calcineurin inhibitors (Bekersky et al., 2001a, Pirsch et al., 1997). Tacrolimus binds to an immunophilin, FK506 binding protein (FKBP) which binds to and blocks CaN. This complex inhibits the activation of NF-ATc, thus preventing its entrance into the nucleus and T-cell activation (Fleischer, 1999). Tacrolimus has demonstrated efficacy both as primary immunosuppressive therapy in patients undergoing various transplantation procedures and as a rescue therapy for patients with refractory acute allograft rejection after liver or kidney transplantation. It is up to 100 times more potent than CsA and clinically, it is associated with a greater reduction in the incidence of acute and chronic rejection and better long term graft survival (Tsunoda and Aweeka, 2000). The common adverse events of tacrolimus are nephrotoxicity, neurotoxicity, and diabetogenicity, which correlate with trough blood concentrations of tacrolimus. Hypertension, hyperkalemia, and thrombotic microangiopathy have also been reported. Neurotoxicity, alopecia, and NODAT develop more frequently with tacrolimus than with ciclosporin. However, tacrolimus is less likely to cause hyperlipidaemia, hypertrichosis and gingival hypertrophy compared with ciclosporin (Prescilla, 2013).

Tacrolimus gastrointestinal absorption is incomplete and variable with an oral bioavailability of approximately 25%. The rate and extent of tacrolimus absorption is greatly affected by food. Tacrolimus binds extensively to erythrocytes in blood and approximately 99% of tacrolimus partitioned into plasma is bound to plasma proteins, mainly albumin and alpha-1-acid glycoprotein. The unbound fraction of tacrolimus, comprising less than 0.1% of total blood concentration, is responsible for its pharmacological activity. Tacrolimus is extensively distributed in the body and at steady state the majority of tacrolimus exists in the tissue. Tacrolimus is predominantly metabolized by CYP 3A enzymes in the liver and the intestinal wall, with a half-life of approximately 12 hours. Most of the tacrolimus dose is ultimately excreted in faeces (Goodman et al., 2006).

2.1.4.3 Antiproliferative Agents

2.1.4.3.1 Azathioprine (AZA)

Azathioprine (AZA) is an immunosuppressive drug used in organ transplantation and autoimmune diseases and belongs to the chemical class of purine analogues. Azathioprine acts as a pro-drug for 6-mercaptopurine, inhibiting an enzyme that is required for the synthesis of DNA. Thus, it most strongly affects proliferating cells, such as the T lymphocytes and B lymphocytes of the immune system (Maltzman and Koretzky, 2003). AZA is converted to 6-mercatopurine and then to 6-thioinosine monophosphate – a precursor of thioguanine nucleotides which particularly inhibit the synthesis of purines required for DNA and RNA replication. Thus it most strongly affects proliferating cells, such as the T lymphocytes and B lymphocytes of the immune system. Moreover, azathioprine blocks the downstream effects of CD28 co-stimulation, a process required for T cell activation. The main adverse effect of azathioprine is bone marrow suppression. Azathioprine can also result in a decrease in the number of blood cells in the bone marrow, which may cause serious or life-threatening infections. Although not very common, hepatotoxicity has been found to be associated with azathioprine therapy (Patel et al., 2006). Additionally, azathioprine may increase the risk of developing certain types of cancers, especially skin cancer and lymphoma and

has therefore been listed as a human carcinogen in the 12th Report on Carcinogens by the National Toxicology Program of U.S. Department of Health and Human Services (NTV, 2011).

Azathioprine undergoes extensive metabolism to mercaptopurine, which is subsequently metabolized to 6-thioguanine nucleotides that are the presumed active moiety. The maximum concentration is reached within about 1-2 hours after administration and it is moderately bound to plasma protein. The half-life of azathioprine is about 10 minutes and that of the metabolite is 1 hour (Goodman et al., 2006). Thiopurine S-methyltransferase (TPMT) contributes to the metabolism of all thiopurines and is responsible for inactivation of mercaptopurine. TPMT shows genetic polymorphism causing null or decreased enzyme activity. Patients who have two nonfunctional TPMT alleles commonly experience life-threatening myelosuppression. TPMT genotyping or phenotyping prior to starting treatment with azathioprine may help in identifying patients at increased risk for toxicity and is recommended by several guidelines (Dean, 2012).

2.1.4.3.2 Mycophenolate Mofetil (MMF)

Mycophenolate mofetil (MMF), a prodrug of mycophenolic acid which is also available as an enteric-coated sodium salt (MPS), is used extensively in transplant medicine (Meier-Kriesche et al., 2011). MMF is an organic synthetic derivative of the natural fermentation product mycophenolic acid (MPA) that causes non-competitive reversible inhibition of inosine monophosphate dehydrogenase, a key enzyme in the *de novo* guanosine nucleotide synthesis pathway in B and T lymphocytes. This interferes with purine synthesis and prevents proliferation of T and B lymphocytes (Allison and Eugui, 2000). One of the essential properties of mycophenolate is that it lacks cardiovascular risks and chronic nephrotoxic adverse effects. The most common adverse events are primarily gastrointestinal such as nausea and/or vomiting, diarrhoea, gastritis, duodenitis, oesophagitis and ulcers. Other adverse effects are related to bone marrow suppression, including leukopaenia, anaemia, and thrombocytopaenia (Prescilla, 2013).

Mycophenolate mofetil is rapidly absorbed from the gastrointestinal tract after oral administration and it is rapidly and completely metabolized to mycophenolic acid. MPA, in turn, is metabolized in the liver, gastrointestinal tract and kidney to the inactive metabolite, 7-O-MPA-glucuronide (MPAG) with a small proportion metabolysed to an acyl-glucuronide. MPAG is actively transported into bile and passes into urine. MPAG is de-conjugated back to MPA by the bacteria in the gut and then reabsorbed in the colon resulting in a second peak of absorption. MPA is highly bound to serum albumin and it reaches its maximum concentration within 1–2 hours (Staatz and Tett, 2007).

2.1.4.4 Sirolimus

Sirolimus is a potent immunosuppressive drug widely used in organ transplantation. It is a macrocyclic triene antibiotic produced by Streptomyces hygroscopicus. Sirloimus was first isolated as an antifungal drug. However, further studies showed its remarkable antitumor and immunosuppressive activities. Sirolimus has a potent inhibitory effect on antigen-induced proliferation of T and B lymphocytes, and inhibits antibody production. Sirolimus binds to the same intracellular protein as tacrolimus, FKBP12. The sirolimus: FKPB12 complex interacts with and blocks the activation of an essential cell-cycle kinase named mTOR (mammalian target of rapamycin). By interfering with the mTOR function, sirolimus prevents the mTOR-mediated signal-transduction pathways and consequently, this results in the blockage of cell-cycle progression in G1 phase (Sehgal, 2003). The most common adverse effects attributed to sirolimus were hyperlipidemia, infection, lymphopenia, anemia, thrombocytopenia, mouth ulcers, peripheral oedema and diarrhoea. Sirolimus -induced pneumonitis has also been reported. In contrast to CNIs, sirolimus is not associated with nephrotoxicity. Therefore, it can be used as an alternative immunosuppressive agent for transplant recipients who develop renal impairment with ciclosporin or tacrolimus therapy (Knechtle, 2014). Sirolimus is rapidly absorbed from the gastrointestinal tract following oral administration and reaches its peak concentration in 1-2 hours. After absorption, sirolimus is extensively bound to red blood cells and less than 5% of the drug remains free in the plasma. Sirolimus has a relatively low bioavailability (around 25%). It is metabolized in the liver and the intestinal wall by the CYP3A enzyme subfamily (CYP3A4 and CYP3A5)

and to a minor extent by CYP2C8. Sirolimus has a long elimination half-life of about 60 hours in renal transplant recipients. It has a narrow therapeutic index and theraputic drug monitoring of blood concentrations is essential to ensure safe and effective treatment (Knechtle, 2014).

2.1.4.5 Antibody Immunosuppressive Therapy

2.1.4.5.1 Monoclonal Antibody

A series of monoclonal antibodies (mAbs) are commonly used as induction therapy in renal transplantation. Monoclonal antibodies have different mechanisms of action. They target specific CD proteins on the T or B lymphocyte surface, including mAbs against CD3, CD20 and CD52.

Alemtuzumab (also known as Campath) binds to CD52, a protein present on the surface of mature lymphocytes, leading to lymphocyte lysis via complement activation and antibody dependent cellular cytotoxicity, leading to depletion of T and B lymphocytes in the peripheral circulation for several months after administration. Infusion related side effects can occur during or shortly after alemtuzumab infusion, including pyrexia, chills/rigors, nausea, hypotension, urticaria, dyspnoea, rash, emesis, and bronchospasm.

Rituximab binds to CD20, a protein present on the surface of immune system B lymphocytes, the combined effect results in the elimination of B lymphocytes.The most common complications occurring with rituximab therapy is infection, pulmonary toxicity, neutropenia and thrombocytopenia (Mahmud et al., 2010, Zaza et al., 2014).

2.1.4.5.2 Polyclonal Antibody

The polyclonal antibodies are produced by immunizing animals with human lymphocytes and the created antilymphocyte serum is then purified to isolate antithymocyte globulins (ATGs) from horses (equine antithymocyte globulin (eATG)) or rabbits (rabbit-derived antithymocyte globulin (rATG)). Polyclonal antibodies prevent T lymphocyte activation and proliferation by its broad spectrum activity against T lymphocytes activation markers, including CD2, CD3, CD4, CD8, CD11a, CD18,

CD25, CD44, CD45, human leukocyte antigen (HLA). Polyclonal antibodies block T-lymphocytes membrane proteins, causing alteration of the function, lysis, and prolonged T- lymphocyte depletion. By using ATGs, the body identified the transplanted organ as non-foreign. In the last decade, rATG has been used as by more transplant centres as an induction agent. The elimination half-life is 2 to 3 days for rATG and 1.5 to 12 days for eATG. The most common side effects of polyclonal antibodies with first-dose infusion are fever, chills and rashes. Repeated doses are associated with myelosuppression, thrombocytopenia and leukopenia. However, these side effects generally diminish after lowering the dose or terminating the treatment (Mahmud et al., 2010).

2.1.4.6 Belatacept

Belatacept is the first biologic agent approved by FDA for maintenance immunosuppression in kidney transplantation combined with other immunosuppressants such as basiliximab, mycophenolate mofetil and corticosteroids in adult patients. Belatacept has comparable short-term outcomes to the CNIs, while avoiding their nephrotoxic potential and their cardiovascular and metabolic toxicities. It is a selective T-lymphocyte costimulation blocker. Belatacept binds to the B7-1/CD80 and B7-2/CD86 receptors of antigen-stimulating cells and thereby prevents the CD28-mediated T-lymphocyte costimulation resulting in the inhibition of IL-2 expression and hence the blockade of T- lymphocyte activation. The most common adverse effects of belatacept include infection, hypophosphatemia, diarrhoea, and cough. The most serious side effect during belatacept therapy are post-transplant lymphoproliferative disorder and progressive multifocal leukoencephalopathy. Belatacept is administered as an intravenous infusion over 30 minutes. It exhibits linear pharmacokinetics. Belatacept has a consistent and predictable pharmacokinetic profile and the serum elimination half-life is approximately 8-10 days. Belatacept has low variability in its exposure and therefore therapeutic drug monitoring is not required (Melvin et al., 2012).

2.1.5 Therapeutic Index and Therapeutic Drug Monitoring

The therapeutic index (TI), which is also known as therapeutic window or range —is defined as the ratio of the dose that produces toxicity to the dose that produces the clinically desired or effective response in a proportion of individuals (**Figure 3)**. Hence, TI is an important indicator in efforts to achieve the safety–efficacy balance (Finkel et al., 2009).

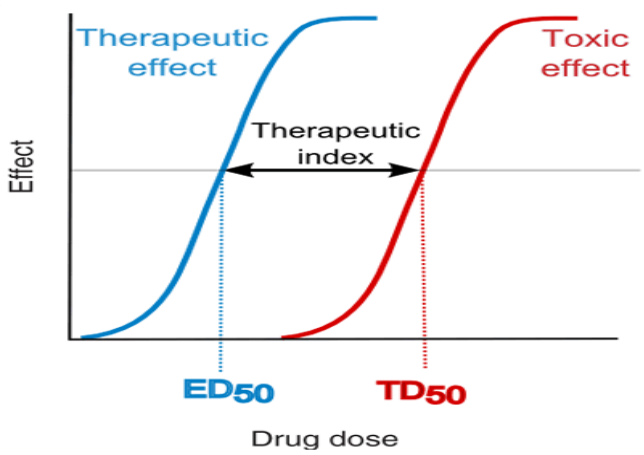


Figure 3: Therapeutic Index (Guzman, 2014). Therapeutic index (TI) = TD50/ED50. ED50 = the drug dose that produces a therapeutic or desired response in half the population. TD50 = the drug dose that produces a toxic effect in half the population.

The therapeutic index varies widely among substances. Drugs which offer a wide therapeutic index are considered very safe. Patients have to take much higher doses of these drugs to reach the toxic levels. On the other hand, drugs with a narrow therapeutic range have little difference between toxic and therapeutic doses. Small changes in systemic exposure can lead to marked changes in their pharmacodynamic responses. Therefore, it is important to adjust the dose by measuring the plasma or blood concentration of these drugs for each individual patient to avoid the unnecessary administration of the drug and to maximise its efficacy. This may be achieved through therapeutic drug monitoring (TDM). Therapeutic drug monitoring enables drug dosage individualization by maintaining the plasma or blood concentrations within the targeted

therapeutic window and consequently, achieving the maximum efficacy and safety of a particular medication (Kang and Lee, 2009).

Most immunosuppressant drugs are critical dose drugs with a large between- and within- individual variability in their pharmacokinetics (PK) and systemic exposure. The between-patient variability is the variation between individuals in the blood concentration achieved by a given dose. On the other hand, the within-patient variability is visualized by fluctuating concentration of the immunosuppressive drug within a certain period of time during which drug dosage was unchanged. High within-patient variability complicates therapeutic drug monitoring as the drug concentrations will frequently be above or below the therapeutic window, putting the patient at risk for toxicity in the case of overexposure or for acute rejection in the case of drug concentrations below the lower threshold of the therapeutic window.

The between-patient variability of calcineurin inhibitor concentrations can lead to under- or over-immunosuppression putting some patients at risk for rejection or toxicity and hence it is an important risk factor for poor kidney allograft outcomes and to some extent this is influenced by genetic factors (Staatz and Tett, 2004). For both CNIs, most of the between- and within-patient variability occurs in the absorption phase rather than in the elimination phase. Individual variation in response to these drugs is a major medical issue and to a certain extent it is genetically related (Schiff et al., 2007). Therefore, regular monitoring and regulation of immunosuppressant drug doses is a crucial indicator to prevent possible rejection events and major adverse effects associated with toxic and sub-therapeutic drug concentrations. Efficacy and side effects of immunosuppressive drugs are highly correlated with the area under the concentration time curve (AUC). Recording a complete pharmacokinetic profile for every patient is not feasible in clinical practice (Op den Buijsch et al., 2007). Limited sampling strategy (LSS) including a limited number of samples such as trough and time points within a short time post-dose, offers a practical approach to investigate the potential of drug AUC monitoring in routine clinical application (Mathew et al., 2008). Some of the common TDM techniques include the following:

2.1.5.1 Trough Concentration Monitoring (C_0)

In this method, the drug concentration that is achieved just before the administration of the subsequent dose is measured. This method is the oldest and, most commonly used method applied in TDM. Despite the simplicity of this method, it provides just an approximate estimate of the drug exposure throughout the dosing interval which is considered as the major downsides of this technique (Norman and Turka, 2001). Whereas the advantages of C_0 monitoring are that it requires only one sample and eliminates the patients need to stay long time in the hospital for multiple samples to be taken.

2.1.5.2 Area under the Curve (AUC) Monitoring

This method provides a more accurate measure of drug extent. $AUC_{0\text{-}12}$ is a good indicator for measuring drug exposure but the main disadvantages of AUC monitoring are that it is expensive and requires multiple blood samples and patient requires to spend long hours in the hospital which is impracticable for both the clinician and the patient.

2.1.5.3 Abbreviated Area under the Curve Monitoring

The AUC monitoring method can be modified by targeting the blood concentration in the first four hours after dosing. This abbreviated AUC is a limited sampling (2-4) strategy over the dosing interval, making it more convenient and economical than the standard AUC monitoring (Norman and Turka, 2001).

2.1.5.4 C2 Monitoring

This method further limits the number of collected samples to one, 2 hours following the drug administration. It is a more accurate predictor of ciclosporin exposure and clinical events as compared to C_0 monitoring as most of the between patient variability is in the absorption rather than the elimination phase (Jorga et al., 2004).

2.2 Introduction on Bioequivalence and Genotyping Studies

2.2.1 Bioequivalence-Narrow Therapeutic Index Drugs

Pharmacokinetics includes the kinetic study of drug absorption, distribution, metabolism, and elimination (ADME). Bioequivalence studies are the commonly accepted method to determine the therapeutic equivalence between two pharmaceutical products. Bioequivalence studies are normally performed using two-way crossover design on healthy adult subjects, aged between 18-25 years, with the aim to minimise variability and detect the differences between the drugs under the conducted study (FDA., 2003). Crossover design is a design in which each group receives different treatments at different dosing periods (**Figure 4**).

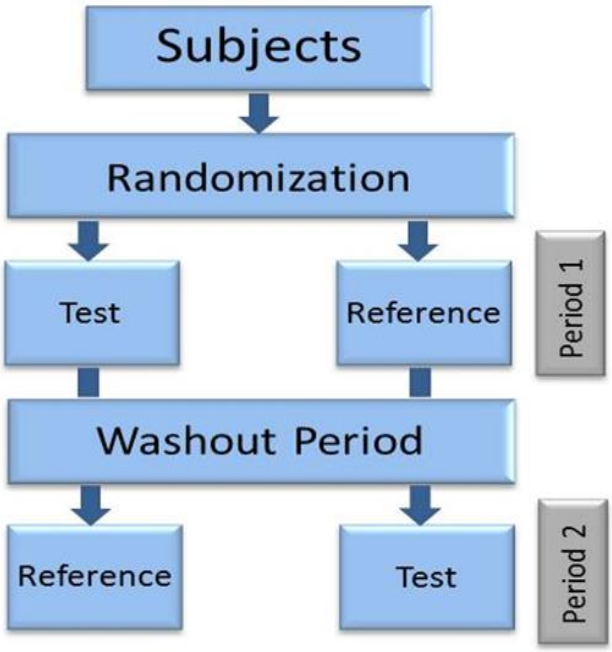


Figure 4: Two-period Crossover Design. At period 1, members of one group receive a single dose of the test formulation and members of the other group receive a single dose of the reference formulation. This is followed by a washout period during which subjects receive no treatment. At period 2, members who receive the test formulation receive the reference formulation and vice versa.

The advantage of the crossover design is that it decreases the influence of between-subject variability as each patient serves as his own control. There are two disadvantages of this design. The first disadvantage is that the treatment order may affect the outcomes. The second disadvantage, which is of the main concern, is the carry-over effect between the treatments that can be avoided with a sufficiently long "wash-out" period (Wang and Bakhai, 2006). This washout period should be equal to or more than five times of the drug elimination half-life, to ensure that drug concentrations are below the lower limit of bioanalytical quantification in all subjects at the beginning of the second period (FDA., 2003).

Bioequivalence of two formulations of the same drug includes equivalence with respect to the rate and extent of absorption. The area under the concentration time curve (AUC) reflects the extent of exposure. The maximum plasma concentration (C_{max}) and the time to maximum plasma concentration (t_{max},) are parameters that are influenced by the absorption rate. FDA guidelines states that the 90% confidence intervals for the ratios (test: reference) of the areas under the drug concentration versus time curves (AUC ratio) and the maximum plasma drug concentrations (C_{max} ratio) must fall between 0.8-1.25, which is the bioequivalence acceptable range applied for any bioequivalence study (FDA., 2003).

Many countries have established guidelines for the bioequivalence studies and formulated requirements for their design, conduct, and evaluation. However, all of these bioequivalence guidelines approved that the acceptable bioequivalence range for the bioavailability measures (AUC and C_{max}) is 0.8-1.25. However, in recent years there have been large debates regarding the validity of this target interval for products with a narrow therapeutic index. It is sometimes claimed that the 80 to 125% limit means there can be a wide variation between the generic and the reference product (Birkett, 2003).

Drugs with a narrow therapeutic index (NTI-drugs) are drugs with small differences between therapeutic and toxic doses. Moreover, there is a risk of clinically relevant difference in efficacy or safety between two NTI drug formulations even when the conventional criteria for bioequivalence (i.e. 90% confidence interval for test / reference

ratio for AUC and C_{max} within 80-125%) are met (Borgheini, 2003, Burns, 1999). Several immunosuppressive drugs, including tacrolimus are considered to be examples of NTI drugs (Johnston and Holt, 1999, MacPhee et al., 2002). For this reason, the Health Canada guidelines were changed for critical dose drugs so that 90% confidence interval of relative mean AUC of the test to reference formulation were reduced to 90.0 to 112.0%, but it is still 80.0 to 125.0% for relative mean C_{max}. European Medical Agency guideline (EMA) was recently changed to recommend that in specific cases of products with a narrow therapeutic index, the acceptance interval for AUC should be tightened to 90.00-111.11%. Where C_{max} is of particular importance for safety, efficacy or drug level monitoring the 90.00-111.11% acceptance interval should also be applied for this parameter (EMA, 2010a). In June 2015, the Efficacy Working Party (EWP) recommended that the bioequivalence acceptance criteria for tacrolimus should be [90-111%] for AUC and [80-125%] for C_{max}. This is because they considered the total drug exposure (AUC) is the key parameter of importance for tacrolimus dose titration; in comparison peak whole blood concentration does not seem to be critical for either safety or efficacy (EMA, 2015). Furthermore, NTI drugs often have steep concentration response relationships for efficacy, toxicity, or both. Dosing generally needs to be individualised based on blood/plasma concentration monitoring and there may be potentially serious clinical consequences in the event of concentrations outside the therapeutic window. Remarkably, it is not possible to define a set of criteria to categorize drugs as either NTIDs or not and a judgment must be made in each individual case. Likewise, the need for narrowing the acceptance interval for both AUC and C_{max} or for AUC only should be determined on a case by case basis (NTV, 2011).

2.2.2 Genotyping Procedure

A gene is a chromosomal DNA sequence that is required for the making of a functional product (polypeptide or functional RNA molecule). An "allele" is one variant of the gene. SNPs or single nucleotide polymorphisms are alterations in a single base in the gene sequence and may code for a different protein altogether creating genetic mutation. Mutations of genes that are important for functions in the body can lead to a genetic condition that may affect the individual growth or health (Mandal, 2014). Some genes

are highly polymorphic, resulting in enzyme variants that may have variable drug-metabolizing capacities among individuals. Many of the recorded polymorphisms relate to differences in the expression of drug metabolizing enzymes and transporters, and hence drug disposition (Flanagan et al., 2007).

Genotyping is the process of determining differences in an individual genetic make-up by examining the individual's DNA sequence using biological assays and comparing it to another individual's sequence or a reference sequence. Two steps are required for single nucleotide polymorphisms (SNPs) genotyping. The first step is DNA region amplification, approximately 200 base pairs around the SNP, millions to billions of times by means of a PCR. Then the PCR product has to be sequenced or digested by an enzyme and followed by gel electrophoresis in order to genotype the polymorphic regions.

2.2.2.1 Polymerase Chain Reaction (PCR) and DNA Sequencing

2.2.2.1.1 Principle of PCR

Polymerase chain reaction (PCR) is a biochemical technology in molecular biology and it is used to amplify a specific region of a DNA strand in a cyclic process to make a huge number of identical copies that can readily be analysed. This is necessary to have enough starting template for sequencing. PCR is commonly carried out in a thermal cycler. The thermal cycler heats and cools the reaction tubes to achieve the temperatures required at each step of the reaction in a very short time.

I. **Initialization Step:** This step consists of heating the reaction to a temperature of 94–96°C (or 98°C if extremely thermostable polymerases are used), which is held for 1–9 minutes. It is only required for DNA polymerases that require heat activation by hot-start PCR (Sharkey et al., 1994).

II. The Cycling Reactions:

There are three major steps in a PCR, which are repeated for 30 or 40 cycles (**Figure 5**).

- **Denaturation Step:** This step is the first regular cycling step and consists of heating the reaction to 94–98 C for 20–30 seconds. During the denaturation, the double strand DNA and primers melt to a single stranded DNA by disrupting the hydrogen bonds between complementary bases and all enzymatic reactions stop.
    - **Annealing Step**: During this step, the reaction temperature is lowered to 50–65°C for 20–40 seconds and this allows annealing of the primers to the single-stranded DNA template. The primers are moving randomly and stable DNA-DNA hydrogen bonds are only formed when the primer sequence very closely matches the template sequence. The polymerase binds to the primer-template hybrid and begins DNA formation.
- **Extension/Elongation Step:** The temperature at this step depends on the DNA polymerase used and commonly a temperature of 72°C is used with this enzyme. At this step the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding dNTPs (deoxynucleotide triphosphates) that are complementary to the template in 5' to 3' direction, condensing the 5'-phosphate group of the dNTPs with the 3'-hydroxyl group at the end of the nascent (extending) DNA strand (Chien et al., 1976). The extension time depends both on the DNA polymerase used and on the length of the DNA fragment to be amplified. Normally, at its optimum temperature, the DNA polymerase will polymerize a thousand bases per minute. At each extension step, the amount of DNA target is doubled, leading to exponential (geometric) amplification of the specific DNA fragment.
- **Final Elongation:** This single step is occasionally performed at a temperature of 70–74°C for 5–15 minutes after the last PCR cycle to ensure that any remaining single-stranded DNA is fully extended.

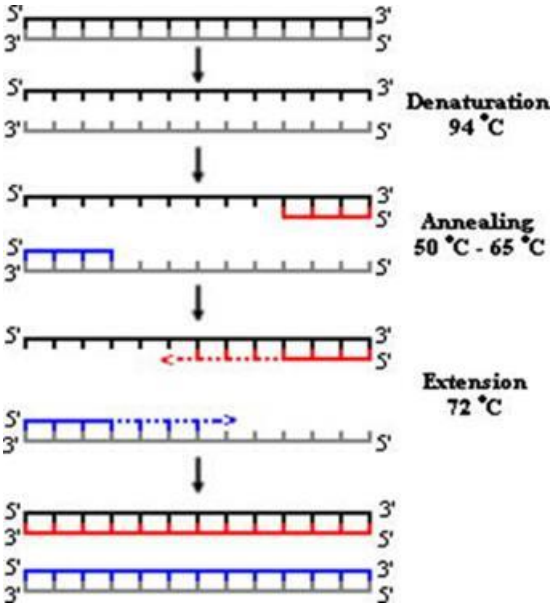


Figure 5: The Different Steps of Polymerase Chain Reaction (Lee et al., 2007). The double strand DNA is denatured by heating to 94-98°C. At approximately 55°C, the primers anneal to the single-stranded DNA template. At 72°C DNA polymerase extends the single-stranded DNA and synthesizes a new DNA strand complementary to the DNA template. This process is repeated for 30 or 40 cycles.

In order to check whether the PCR generated the anticipated DNA fragment, agarose gel electrophoresis is employed for size separation of the PCR products. The size of PCR products is commonly determined by a comparison with a DNA ladder (a molecular weight marker), which contains DNA fragments of known size; run on the gel alongside the PCR products. The products with brighter fluorescence are the ones that are likely to produce the best sequences.

2.2.2.1.2 Principle of Sequencing

The purpose of sequencing is to determine the order of the nucleotides of a gene from PCR fragments or cloned genes. There are three major steps in a sequencing reaction (like in PCR), which are repeated for 30 or 40 cycles.

- **Denaturation Step at 94-98°C**: During the denaturation, the double strand DNA and primers melt to a single stranded DNA by disrupting the hydrogen bonds between complementary bases and inhibiting enzymatic reactions.

- **Annealing Step at 50-65°C:** In sequencing reactions, only one primer is used, so there is only one strand copied (in PCR: two primers are used, so two strands are copied). During this step, this lowered temperature allows annealing of the primer to the single-stranded DNA template. The primer is moving randomly around and stable DNA-DNA hydrogen bonds are only formed when the primer sequence very closely matches the template sequence. The polymerase binds to the primer-template hybrid and begins DNA formation.
- **Extension Step at 60°C:** At this step the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding dNTPs or ddNTPs (dideoxynucleotide triphosphates) that are complementary to the template in 5' to 3' direction. Normally, the ideal working temperature for the polymerase is 72°C but because it has to incorporate ddNTPs which are chemically modified with a fluorescent label, the temperature is lowered so it has time to incorporate the 'strange' molecules. When a ddNTP is incorporated, the extension reaction stops because a ddNTP contains a H-atom on the 3rd carbon atom compared to dNTPs that contain a OH-atom on that position). Since the ddNTPs are fluorescently labelled, it is possible to detect the colour of the last base of this fragment on an automated sequencer (**Figure 6**).

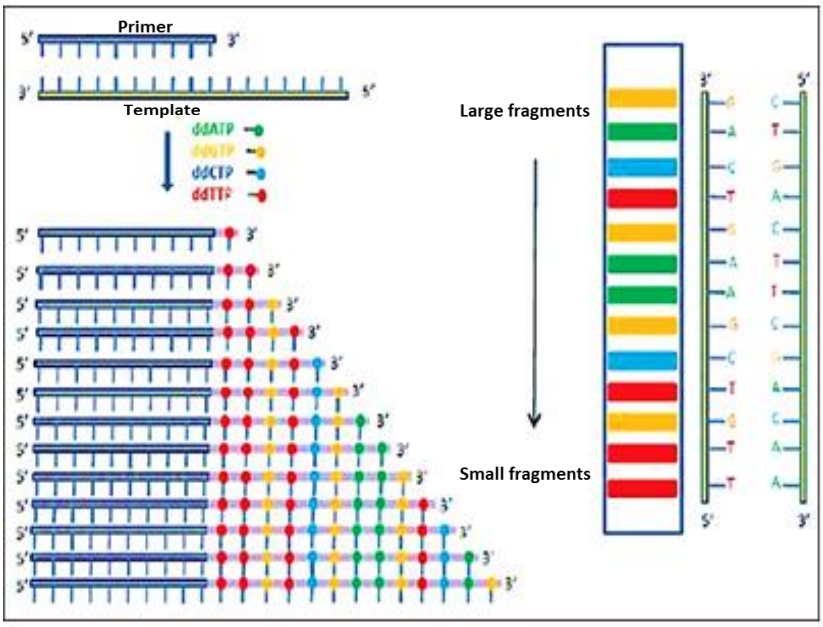


Figure 6: Chain Termination Sequencing Showing the Different Lengths of Labeled DNA strands (Aliyu, 2014).

Sequencing reactions need to be cleaned up prior to injection on the ABI sequencer in order to remove both the unincorporated dideoxynucleotides as well as salts from the reaction buffers. This is achieved by filtration on sephadex column (Hutchison et al., 2005). Addition of reagents such as formamide may increase the specificity and the yield of PCR (Sarkar et al., 1990).

After the sequencing reactions, a mixture of strands with different length and ending on a fluorescently labelled ddNTP has to be separated. This can be done with gel electrophoresis using a fluorescence-based capillary electrophoresis (CE) system. When the DNA passes through the detection cell, the capillaries are simultaneously illuminated from both sides of the array by a laser beam. The fluorescent molecule sends out a light of a distinct colour. The emitted fluorescent light is collected, separated by wavelength, and focused onto a charge coupled device (CCD). This is followed by data transfer to the instrument computer where they are transformed by chemometric algorithmic processing into 4- or 5-dye electropherograms. Each base has its own colour, so the sequencer can detect the order of the bases in the sequenced gene, see **Figure 7**.

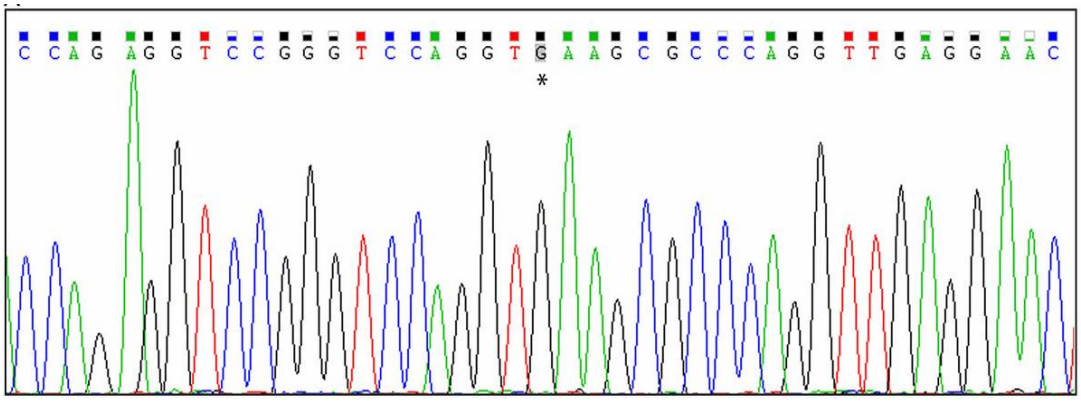


Figure 7: An Electropherogram Showing a Sequence of Data Produced by an Automated DNA Sequencing Machine (Abouzeid et al., 2012). The four dideoxy nucleotides (A, G, C and T) have different fluorescent colours. A base =green colour, C base= blue colour, G base = black colour and T base= red colour. The DNA sequence can easily be identified by using these colour codes.

2.2.2.1.3 Real-time PCR (RT-PCR)

Real-time PCR is a quantitative PCR technique in molecular genetics that follows the principle of PCR with simultaneous amplification and detection of specific DNA-sequences. It is an established method for DNA quantification that measures the accumulation of DNA product after each round of PCR amplification and it monitors the progress as it occurs, in real time (Higuchi et al., 1992, Higuchi et al., 1993). Consequently, it allows quantification of the target polymorphic DNA regions and genotyping of single nucleotide polymorphisms (SNPs) in one single PCR run. RT-PCR facilitates a high speed PCR analysis, about one hour/run. It also minimizes the risk of contamination during analysis, since no post PCR steps are involved and the PCR product remains in the machine for genotyping (Reuter et al., 2005).

Melting curve analysis can only be implemented with real-time PCR detection technologies where the fluorophore, a fluorescent compound, remains linked to the target DNA sequence. Hybridization probes, sequence-specific oligonucleotides, are required for DNA amplification process and genotyping using melting curve analysis. The probes are usually 100-1000 bases long, labelled by fluorescence dyes; one probe is labelled on the 3' end and the other is labelled on the 5' end. The 3' end contains a phosphorylation modification so that the probe is not participating in extension (**Figure 8)**. The probe that binds to the DNA strand and covers the polymorphic region, the predicted site of mutation, is called the sensor hybridisation probe and the probe that binds to a site with only 1–5 bases distance from the sensor probes is called the anchor probe that produces the fluorescent signal. The sensor probe is labelled by donor fluorophore and the anchor probe by acceptor fluorophore (Reuter et al., 2005, Broude, 2002).

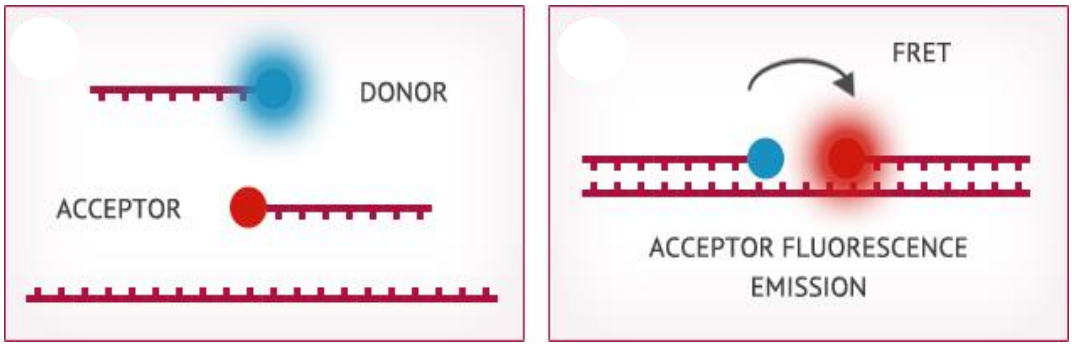


Figure 8: Hybridization Probes. During annealing the two probes bind to their target sequences and an energy transfer occurs within the donor - acceptor pair. The increase in fluorescent activity of acceptor fluorophore is measured and the fluorescence signal is directly proportional to the target DNA amount.

The primers amplify the target sequence and these two allele-specific probes are designed to hybridize to the target sequence only if they are perfectly matched. When the probes hybridize to their target sequence, they become fluorescent. During amplification the hybridisation probes anneal to the amplified DNA segment in a head to tail configuration and this separates the two fluorophores from each other. A fluorescence resonance energy transfer (FRET) can occur between them, providing real-time monitoring of the amplification process. By means of a light-emitting diode (LED), energy transfer begins when the light source excites the donor fluorophore, the fluorescent dye of the sensor probe which then emits fluorescent light. Through FRET, the energy of that light in turn excites the acceptor reporter fluorophore in the anchor probe which then emits another fluorescent light. The optical unit of RT-PCR machine measures the intensity of the emitted light. The energy transfer is referred to as FRET. The FRET signal becomes stronger when more new copies of the target DNA sequences are produced and more probes hybridise to the DNA. Consequently, the FRET signal represents the amount of DNA copies in a PCR run. During the PCR elongation phase, the hybridization probes set free from the DNA as the temperature increases. After PCR completion, melting-curve analysis can be used to detect the point mutations. Fluorescence can be monitored as the temperature slowly elevated from 40°C to 75°C through the melting temperature (Tm), where the hybridization probes are melted off the DNA strand and the presence or absence of a mutation can be detected by the obtained characteristic melting profile for each genotype. If the sensor probe is

completely annealed to the DNA strand, it melts off at a higher temperature than the lower temperature required to melt off a probe with a mismatch of one base, single nucleotide mutation. The melting curve analysis can show three characteristic curves: a curve with a single early peak (homozygous mutant, Mut), a curve with a single late peak (homozygous wild type, Wt) and a curve with two peaks (heterozygous genotype, Wt/Mut); see **Figure 9** (Reuter et al., 2005).

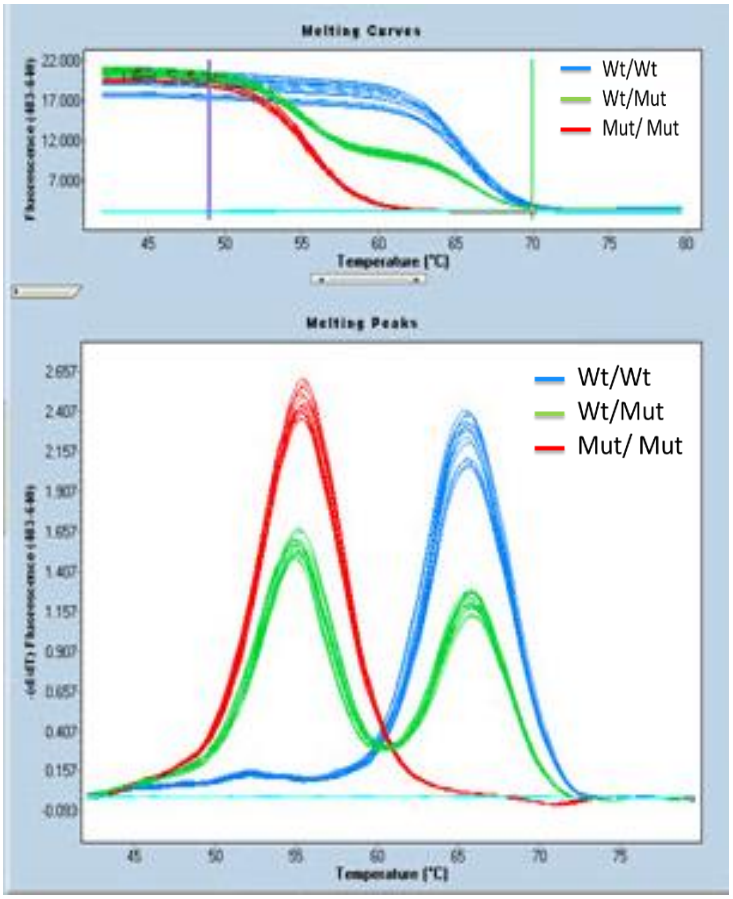


Figure 9: Melting Curve Analysis for Hybridization Probes Designed for Wild Type DNA Sequence. The early and late single peaks indicate homozygous mutant (Mut/Mut) and homozygous wild (Wt/Wt) samples, respectively. The dual peak indicates heterozygous samples (Wt/Mut).

Chapter 3. Pharmacokinetics and Pharmacogenomics of Tacrolimus: A Review

Tacrolimus is a natural macrolide lactone antibiotic with potent immunosuppressive properties (**Figure 10**). Its molecular formula is $C_{44}H_{69}NO_{12}$ with a molecular weight of 804.02 g/moL. Although structurally unrelated to Ciclosporin (CsA), its mode of action is similar. Tacrolimus is considered as one of the most potent immunosuppressive agents used in the prevention of rejection following renal transplantation (Pirsch et al., 1997).

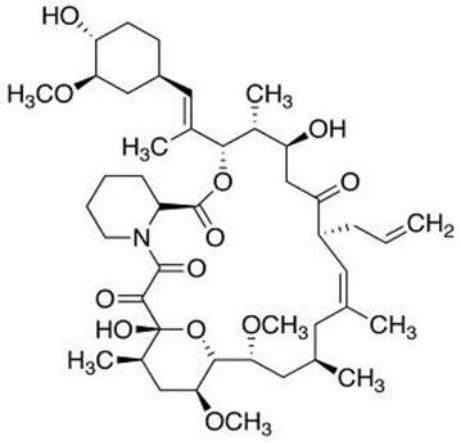


Figure 10: Structure of Tacrolimus.

Tacrolimus acts as an immunosuppressant via inhibiting the proliferation and activation of CD4+ T helper cells through binding to a cellular receptor known as FK506-binding protein (FKBP). The tacrolimus-FKBP complex further binds to calcineurin (CaN), preventing the dephosphorylation of the nuclear factor of activated T cells (NF-ATc). The cascade of cytokine gene transcription is then blocked by inhibiting NF-ATc transport to the nucleus and preventing its binding to the nuclear component of the nuclear factor of activated T cells (NF-ATn). Consequently, T cells do not produce IL-2, which is necessary for full T-cell activation (**Figure 11**). The net result is the inhibition of T-lymphocyte activation: immunosuppression (Fleischer, 1999).

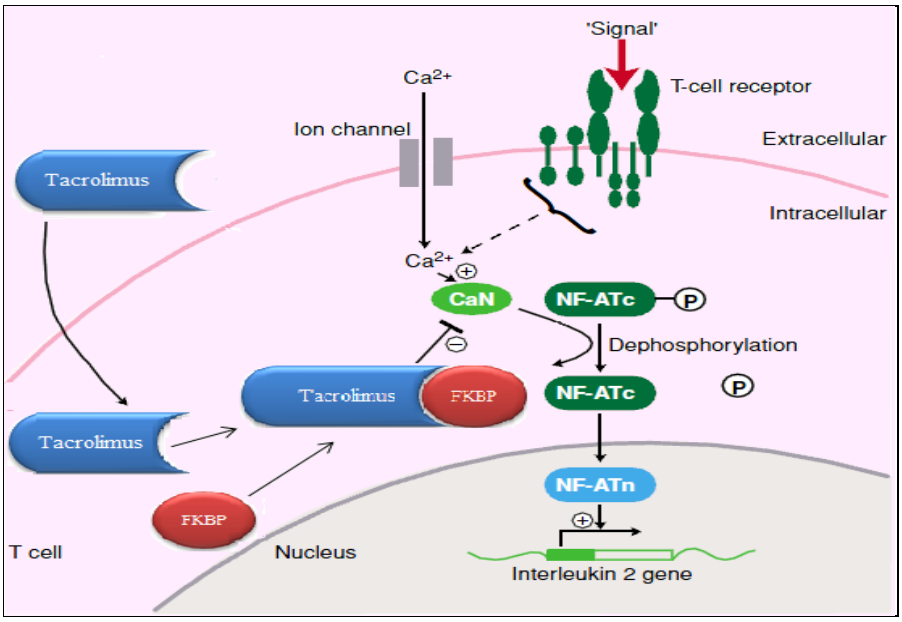


Figure 11: Mechanism of Action of Tacrolimus (Stepkowski, 2000). In the cytoplasm, tacrolimus (FK506) binds to FK506-binding protein (FKBP). The FK506–FKBP complex binds to and blocks the function of the enzyme calcineurin (CaN), which has a serine/threonine phosphatase activity. Accordingly the FK506–FKBP–CaN complex prevents the cytoplasmic nuclear factor of activated T cells (NF-ATc) dephosphorylation and its transport to the nucleus. Consequently, it inhibits IL-2 production, which is necessary for full T-cell activation. FKBP: FK506-binding protein; CaN: Calcineurin; Ca2+: Calcium; NF-ATc: The nuclear factor of activated T cells (Inside the cytoplasm); NF-ATn: The nuclear factor of activated T cells (Inside the nucleus).

Moreover, tacrolimus also has important anti-inflammatory properties that are apparent through inhibiting the release of mast cell and basophil preformed mediators, down regulating IL-8 receptor expression, decreasing intercellular adhesion molecule-1 (ICAM-1) and E-selectin lesional blood vessel expression. This broad range of the inflammatory inhibition mechanism may reduce antigen recognition and down regulate the entire inflammatory cascade leading to clinical disease (Fleischer, 1999).

Tacrolimus was initially available for over 10 years as a preparation requiring twice daily administration: Prograf®; 0.5, 1 and 5 mg capsules (Pirsch et al., 1997). The initial tacrolimus dosage is usually between 0.1 to 0.2 mg/ kg of body weight per day, given in two divided doses. The maintenance tacrolimus dosing is based on a standard protocol

employing therapeutic drug monitoring with the target whole blood 12 hour post-dose (trough) concentrations, depending on the time since transplantation.

The therapeutic range for tacrolimus trough blood concentrations (C_0) lies between 5 and 15 µg/L with a recent trend towards lower target concentrations based on the SYMPHONY study where the target was 3-7 µg/L and the achieved range was 5-10 µg/L (Ekberg et al., 2007). The current regimen for tacrolimus at St George's employs a target range of 8-12 µg/L during the first 3 months after renal transplantation when the risk of rejection is greatest, dropping to 5-8 µg/L thereafter. A number of generic twice daily tacrolimus preparations have become available recently. A prolonged release preparation, Advagraf®, has been available in the UK since 2007. Advagraf® has equivalent efficacy and safety to Prograf® following once daily dosing. This preparation is licensed in the UK for the prevention of renal allograft rejection and it is available in 0.5, 1, 3, and 5 mg capsules. The aim of developing a once daily preparation was to reduce the frequency of dosing and simplify dosing regimens for kidney, liver, and heart transplant recipients currently using the twice daily tacrolimus formulation (Alloway et al., 2007, Silva et al., 2007). A study assessing the adherence to immunosuppressant regimens in kidney transplant patients demonstrated that once-daily tacrolimus dosing improved patients' compliance (Kuypers et al., 2013). Tacrolimus adherence was improved in the majority of liver transplant patients after switching to once-daily tacrolimus and mainly caused by the lack of the evening dose (Beckebaum et al., 2011). Upon change from Prograf® to Advagraf®, the same total daily dose is given as a single dose in the morning with adjustment of the dose to maintain exposure within the range specified above. Prograf® and Advagraf® should be taken on an empty stomach, either one hour before or two hours after a meal. In Phase II studies on Advagraf, a given dose of Advagraf delivered 90% of the area under the concentration-time curve (AUC) obtained with Prograf (EMEA, 2007).

3.1 Tacrolimus Pharmacokinetics

3.1.1 Absorption

After oral administration, tacrolimus is absorbed rapidly in most patients and reaches its peak plasma/blood concentration in 30 minutes to one hour. While in some patients it is absorbed slowly over a prolonged period, resulting in a flat absorption profile. Tacrolimus has a large variability in the rate of absorption and absolute bioavailability between individuals. It ranges from 5%–93% and approximately 25% of the oral dose is bioavailable due to an active barrier to drug absorption (Venkataramanan et al., 1995). The poor water solubility of tacrolimus and reduced gut motility in transplant recipients is responsible for the poor and erratic absorption of tacrolimus. Since tacrolimus is well-known as a substrate of CYP3A iso-enzymes, its poor bioavailability is to a large extent caused by presystemic metabolism of tacrolimus in the gut wall and liver (Tuteja et al., 2001). Tacrolimus is also known as a substrate of P-glycoprotein (P-gp), a multidrug efflux transporter. P-glycoprotein lowers the intracellular concentration of tacrolimus by pumping the absorbed tacrolimus back out into the intestinal lumen, it is repeatedly transported out of the intestinal mucosa cells and then passively reabsorbed. This extensive presystemic metabolism limits the oral bioavailability of tacrolimus to ~25%. Therefore, it is very likely that the poor and the variable bioavailability of tacrolimus is at least partly caused by the activity of this efflux pump in the intestine and genetic polymorphism of the P-glycoprotein (Hoffmeyer et al., 2000). Polymorphisms, or genetic variations, of these isoenzymes affect the dosage requirement and trough levels of tacrolimus in stable transplant patients (Haufroid et al., 2006). The impact of genetic variations in expression of CYP3A and P-glycoprotein will be discussed in detail below.

Tacrolimus absorption is significantly inhibited by food. A study comparing the influence of food on both the rate and extent of Prograf® absorption in healthy volunteers showed that food in general significantly decreases the rate and extent of tacrolimus absorption and slows it down compared with the fasting state. Relative to the fat content of the food, high-fat meals had more pronounced effect than low-fat meals, see **Figure 12** (Bekersky et al., 2001b).

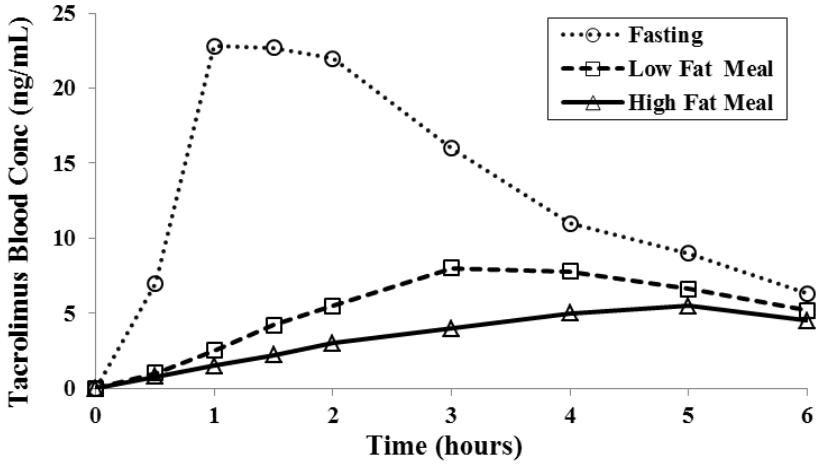


Figure 12: Effect of Food on Tacrolimus Absorption after Administration of 5mg Prograf® (Bekersky et al., 2001b). Food in general and fat in particular, has a clinically significant effect on tacrolimus absorption.

Another study demonstrated that tacrolimus ingestion in the fasting state (10 hours) provides the greatest relative bioavailability in comparison to taking the drug one hour prior to a meal, which had a relatively minor impact on the relative bioavailability extent (~12%). Furthermore, ingestion of tacrolimus immediately after a meal or 1.5 hours subsequent to a meal had a more pronounced influence (**Figure 13**). Hence, the standard recommendation is that tacrolimus must be taken either 1 hour before or 2 hours after eating (Bekersky et al., 2001a).

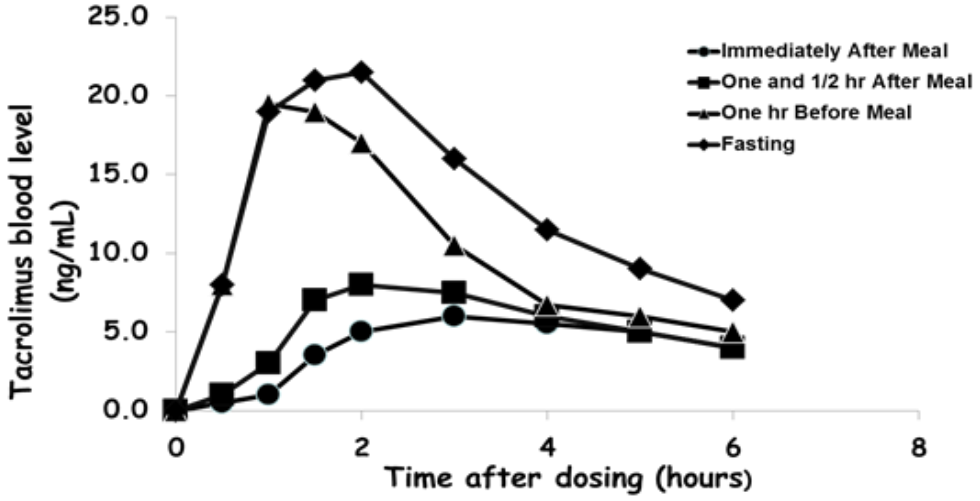


Figure 13: Effect of Time of Meal Consumption on Tacrolimus Absorption after Administration of 5mg Prograf® (Bekersky et al., 2001a). Timing of meals has a significant effect on the rate and extent of drug absorption.

As will be seen in the pharmacokinetic profiles above, while food impacts significantly on the absorption phase and area under the concentration-time curve (AUC), it has relatively little impact on 12 hour post-dose trough concentrations. This is an important practical point as trough concentration monitoring in patients taking tacrolimus along with food may result in false reassurance on drug exposure. Standard guidance is to take tacrolimus either at least one hour before or at least two hours after a meal. Variable compliance with this advice may be one of the factors contributing to within-patient variability in tacrolimus exposure.

Prograf® is predominantly absorbed in the upper part of the gastrointestinal tract; around the stomach and proximal small intestine. Advagraf® is modified to release tacrolimus slowly. Ethylcellulose, hypromellose, and lactose monohydrate was added in order to control water penetration and produce a protective polymer layer around the drug. Due to different dissolution properties, Advagraf® is typically released further along the gut, which provides a more distal area of tacrolimus absorption and this increases the contact interval with the absorption site (Barraclough et al., 2011a).

3.1.2 Distribution

After absorption, tacrolimus is extensively bound to both red blood cells and plasma proteins. In the systemic circulation, tacrolimus binds strongly to erythrocytes in the region of 95% because of its high affinity for the FK-binding proteins which are found abundantly in erythrocytes and lymphocytes. In plasma, approximately 99% is bound to plasma proteins, mainly albumin and alpha-1-acid glycoprotein. The mean tacrolimus whole blood concentration is about 20 times higher than that of plasma. Tacrolimus distribution between plasma and erythrocytes is dependent on several factors including; haematocrit, tacrolimus concentration, plasma protein concentration and temperature at the time of plasma separation (Nagase et al., 1994, Venkataramanan et al., 1995). Less than 0.1% of the unbound fraction of tacrolimus is responsible for its pharmacological activity. Whole blood concentrations of tacrolimus, which consists of both bound and unbound fractions of tacrolimus, are routinely monitored to ensure appropriate exposure to the drug. Tacrolimus is distributed extensively in the body with most partitioned outside the blood compartment (Undre, 2003). Lipophilic drugs such as tacrolimus

readily cross membranes and are taken up by adipose tissue. In animal studies tacrolimus is widely distributed in tissues; lung, spleen, kidney, heart, pancreas, brain, muscle with liver exposed to the greatest accumulation (Burton, 2006)

3.1.3 Metabolism

Tacrolimus undergoes O-demethylation, hydroxylation, or oxidative metabolic reactions. The CYP 3A enzyme system is mainly responsible for tacrolimus metabolism. The CYP3A subfamily consists of four genes, CYP3A4, CYP3A5, CYP3A7, and CYP3A43. Tacrolimus is metabolized predominantly by CYP3A4 and CYP3A5 in the liver and the intestinal wall, with <1% of the parent drug is excreted unchanged in the urine or faeces. CYP3A4 in the gastrointestinal epithelium is responsible for presystemic elimination of approximately 50% of the absorbed dose, whereas first-pass metabolism by CYP3A4 in the liver accounts for an additional 10% of elimination. When CYP3A5 is expressed, it accounts for 50% of the total hepatic CYP3A content (Burton, 2006, Undre, 2003). An *in-vitro* study revealed that CYP3A5 contribution in tacrolimus conversion to its metabolite (13-O-demethyltacrolimus) was higher than CYP3A4 contribution (Kamdem et al., 2005). Another *in-vitro* study reported that CYP3A5 is a more efficient enzyme for tacrolimus metabolism than CYP3A4 (Dai et al., 2006). Moreover, P-glycoprotein inhibits drug absorption from the gut by promoting efflux into the lumen of the intestine; it also has a role in systemic clearance of drugs by promoting efflux into the bile for excretion (Burton, 2006, Undre, 2003).

3.1.4 Elimination

The metabolites of tacrolimus are mainly eliminated via the biliary route and more than 95% of the oral dose was recovered in faeces. Only an average of 2.4% of tacrolimus dose was excreted in urine. However, less than 1% of tacrolimus appears unchanged in urine and stool indicating that tacrolimus metabolism plays a major role in its elimination (Burton, 2006, Dalal et al., 2010).

3.2 Tacrolimus Pharmacogenomics

Genetic differences in drug response may arise from differences in pharmacodynamics or in drug disposition. Many of the recorded polymorphisms relate to differences in the expression of drug metabolizing enzymes and transporters, and hence drug disposition. Some genes are highly polymorphic, resulting in enzyme variants that may have variable drug-metabolizing capacities among individuals. A single-nucleotide polymorphism (SNP) is a DNA sequence variation occurring when a single nucleotide (A, T, C or G) in the genome differs between individuals (Flanagan et al., 2007).

Despite tacrolimus success in improving graft survival, tacrolimus therapeutic use is complicated by its narrow therapeutic index (between 5 and 15 µg/L). The routine therapeutic monitoring of tacrolimus reveals a large between individual variability in tacrolimus pharmacokinetics and dose requirement. Many studies have focused on the possible causes of this variability in tacrolimus pharmacokinetics. Among various factors investigated, genes coding polymorphisms in biotransformation enzymes (CYP3A4/5 isoenzymes) and drug transporters (*ABCB1*, previously known as MDR1) have received much attention (Patel et al., 2012, Haufroid et al., 2006). These genetic polymorphisms may explain the observed variability in tacrolimus pharmacokinetics. They have been studied in relation to tacrolimus dosing and shown to be the most promising. In addition to these genes, a number of recently identified variants have been reported to have a potential influence in tacrolimus pharmacokinetics and dose requirement.

3.2.1 Cytochrome P450 (CYP450) 3A

The cytochrome P450 family (CYP450) is a super-family of related, but distinct, drug-metabolizing enzymes. CYP450s are proteins bound to heme (membrane-bound heme-containing proteins). They are classified on the basis of cDNA cloning according to similarities in amino acid sequence. Members of a subfamily (denoted by a letter) must have at least 55% identity (Flanagan et al., 2007). The most important cytochrome P450 isoforms responsible for drug metabolism are the CYP3A family and mainly present in critical tissues such as liver and the gastrointestinal tract.

The CYP3A family is composed of four enzymes: CYP 3A4, CYP 3A43, CYP 3A5, and CYP 3A7. The expression of CYP3A43 in adult livers is at very low levels, only 0.1–0.2% of CYP 3A transcripts. It does not play a major role in drug metabolism. CYP3A7 is mainly expressed in the foetal liver, but it is expressed in small amounts in some adult livers and extrahepatically. The participation of CYP3A43 and CYP3A7 in CYP3A substrates metabolism in adults is therefore regarded to be negligible (Burk et al., 2002). CYP3A4 and CYP3A5 are highly expressed in the liver as well as extra-hepatic tissues, including the gastrointestinal tract and they have an important role in first-pass metabolism (Flanagan et al., 2007). CYP3A enzymes are estimated to contribute to the metabolism of 40 to 60% of all medicines. In particular, they play a fundamental role in the oxidative, peroxidative, and reductive metabolism of numerous clinically useful therapeutic agents (Burk et al., 2002).

The *CYP3A5* and C*YP3A4* genes are part of a cluster of CYP3A genes located on chromosome 7q22.1 along with other CYP3A family members. The *CYP3A4* gene spans 13 exons covering 27 kb sequence and encodes 502 amino acids with a molecular weight of 57 kDa. CYP3A5 is a 52.5 kDa protein made up of 502 amino acids and encoded by the *CYP3A5*1* allele. *CYP3A5*3* allele is the most common *CYP3A5* variant. CYP3A5 shares 85% sequence similarity with CYP3A4. Both CYP3A4 and CYP3A5 are expressed in the liver and intestine, with CYP3A5 being the predominant form expressed in extrahepatic tissues. CYP3A4 and CYP3A5 have similar substrates (Lamba et al., 2012).

3.2.1.1 CYP3A5

The most frequent variant of functional importance in the *CYP3A5* gene is *CYP3A5*3*. The functional SNP located in CYP3A5 intron 3 (rs776746 A>G), for which A and G nucleotides are designated as *CYP3A5*1* (wild-type) and *CYP3A5*3* (mutant-type) alleles, respectively. *The CYP3A5*1* allele expresses large amounts of CYP3A5, while the *CYP3A5*3* allele creates a cryptic splice site, resulting in a premature stop codon and the absence of functional CYP3A5 (Kuehl et al., 2001). The distribution of *CYP3A5*1* wild type allele is different among ethnic groups and varies in frequency of 5%-15% in Caucasians, 15%-35% in Asians, 25% in Mexicans, and 45%-73% in

African-Americans (Provenzani et al., 2013). The influence of *CYP3A5*3* variant on tacrolimus (Prograf®) metabolism has been extensively studied. It has been reported that CYP3A5 activity explains 29–35% of the variability in the studied tacrolimus pharmacokinetic parameter (de Jonge et al., 2012).

Originally, MacPhee et al. (2002) reported that the variable dose requirement of tacrolimus in kidney transplant recipients was associated with the CYP3AP1 pseudogene. At 3 months after transplantation, patients with a *CYP3AP1*1* allele had twofold lower dose-normalized tacrolimus concentrations than *CYP3AP1*3/*3* homozygotes to achieve target blood concentrations. They also showed that there is an association between CYP3AP1 pseudogene and CYP3A5 expression. *CYP3AP1*1* genotype is tightly linked with *CYP3A5*1*, indicating CYP3A5 expression. While *CYP3AP1*3* allele is in tight linkage with *CYP3A5*3* with no CYP3A5 expression. The authors further confirmed these findings during the first 2 weeks after transplantation in a single centre retrospective study involving data from 178 kidney transplant patients between 1995 and 2001, suggesting a link between the variability in tacrolimus pharmacokinetics and the *CYP3A5*genotype (MacPhee et al., 2004). Since then many studies have been conducted to study the effect of *CYP3A5* genotype on tacrolimus dose and dose-corrected tacrolimus concentration in heart, liver and most extensively in kidney transplantation including healthy volunteers and adult and paediatric patients.

In healthy individuals, a pharmacokinetic study, involving 22 healthy Korean subjects, demonstrated that CYP3A5 expressers yield much lower $AUC_{0-\infty}$ and C_{max} concentrations than the CYP3A5 non-expressers (Choi et al., 2007). This was confirmed by another study in 20 healthy Japanese subjects and the study showed that subjects with *CYP3A5*3/*3* achieved 1.8-fold higher tacrolimus $AUC_{0\text{-}8h}$ than *CYP3A5*1* carriers. Besides, the estimated tacrolimus apparent clearance (CL/F) in *CYP3A5*1* carriers was 1.5 times higher than CYP3A5 non-expressers (Suzuki et al., 2008).

In kidney transplantation, the influence of *CYP3A5* genotype on tacrolimus dose and dose-adjusted concentration has been extensively studied in either adult or paediatric patients. **Table 3** summarises the key tacrolimus pharmacogenetic studies conducted in

renal transplant recipients. It has been consistently demonstrated that recipients carrying at least one *CYP3A5*1* allele have significantly lower tacrolimus dose-adjusted trough concentrations and required 1.5 to 2 times the tacrolimus dose to achieve the same target blood concentrations than those carrying *CYP3A5*3/*3* genotype. These findings have been demonstrated in renal transplant patients with different ethnic backgrounds (Caucasian, African, Japanese, Chinese and Mexican).

In heart transplant patients, the *CYP3A5*3* genotype effect on tacrolimus dose and concentration was similar to that obtained from kidney transplant patients. A study in 65 heart transplant recipients demonstrated that CYP3A5 expressers required higher tacrolimus doses compared with CYP3A5 non-expressers (Díaz-Molina et al, 2012). This was confirmed by a retrospective observational study in 52 heart transplant patients during the first year after transplantation and again showed that CYP3A5 expressers had lower dose-adjusted trough concentrations throughout the study period and required 2.2- to 2.6-fold higher tacrolimus dose to reach the target concentration compared with CYP3A5 non-expressers (Lesche et al., 2014). However, a paediatric study in 38 Jordanian patients found no significant differences in tacrolimus dose, C_0 or C_0/dose among each genotype group of *CYP3A5* polymorphisms in either the early or the maintenance phase after transplantation (Shilbayeh et al, 2013).

Furthermore, *CYP3A5*3* genotype has a strong association with tacrolimus dose and dose-adjusted concentration in liver transplant patients. A study in 216 Chinese liver transplant patients revealed that recipient with *CYP3A5*1/*1* genotype required a higher tacrolimus dose than patients with *CYP3A5*3/*3* allele. Besides, the concentration/dose (C/D) ratio of tacrolimus was lowest in the *CYP3A5*1/*1* than the *CYP3A5*1/*3* and **3/*3* genotypes, indicating that the enzymatic activity of CYP3A5 increased proportionally with the number of *CYP3A5*1* allele (Shi et al., 2013). A retrospective study in 90 Chinese pediatric liver transplant patients showed that despite using the same induction and initial dose of tacrolimus, CYP3A5 non-expressers had a significantly higher C/D ratio than CYP3A5 expressers. CYP3A5 expressers required larger doses during the late induction and the maintained phases to reach the target trough concentration (Chen et al., 2014).

Despite that the *CYP3A5*3* genotype effect on tacrolimus dose and dose-adjusted concentration may differ from that obtained from kidney transplant patients. The reason for this is that the *CYP3A5* genotype of the grafted liver in some cases may differ from the *CYP3A5* genotype of the recipient intestine with the consequences of the relative effect of the donor and recipient genotypes on tacrolimus disposition and when it appears post transplantation.

In all studies where the donor and recipient genotypes are different, the donors' *CYP3A5* gene polymorphisms significantly influence tacrolimus dose and dose-adjusted C_0 and its effect can be found very early from the first week after transplantation or can begin later after transplantation. The recipient genotype effect on tacrolimus dose and dose-adjusted C_0 appears only in some of these studies. Ji et al. (2012) studied 58 Korean adult liver transplant recipients on tacrolimus-based immunosuppression therapy for 4 years of follow-up and reported that throughout the entire study period, CYP3A5 expresser recipients grafted from CYP3A5 expresser donors consistently had the least concentration/dose (C/D) ratio. Whereas CYP3A5 expressed recipients grafted from CYP3A5 non-expresser donors had an intermediate C/D ratio, and CYP3A5 non-expresser recipients grafted from CYP3A5 non-expresser donors had the largest C/D ratio. Another study again showed that CYP3A5 non-expresser recipients who received organs from *CYP3A5*1* allele carriers have a significantly lower C/D ratio than those who received organs from *CYP3A5*3/*3* carriers over 6 months. CYP3A5 expresser recipients who received organs from *CYP3A5*3/*3* carriers have a significantly lower C/D ratio than CYP3A5 non-expresser recipients who received organs from *CYP3A5*3/*3* carriers from 2 weeks through 6 months (Muraki et al., 2011). A retrospective study in 70 Chinese liver transplant recipients and donors during the first three weeks after transplantation found a strong association between tacrolimus C/D ratio and *CYP3A5*3* variant in donors from the first week post-transplant. Tacrolimus C/D ratio was significantly lower in patients engrafted with *CYP3A5*1* carrier liver than those engrafted with *CYP3A5*3/*3* genotype liver (Li et al., 2007). In a study on 50 Chinese liver transplant patients, Wei-lin et al. (2006) reported that at 2 weeks and at one month after transplantation, patients receiving *CYP3A5*1* allele–carrying livers had a higher tacrolimus daily dose or lower tacrolimus C/D ratios compared with patients

receiving the *CYP3A5*3/*3* genotype livers. A study in 60 *de novo* Japanese liver transplant recipients early after transplantation found that the *CYP3A5*1* allele in both donors and recipients had a significant effect on tacrolimus dose and C/D on day 7 posttransplant. After the first month, the *CYP3A5*1* allele in donors, but not in recipients had a significant effect on tacrolimus dose and C/ D ratio. Moreover, patients having the *CYP3A5*3/*3* allele in their native intestine and in the graft liver, showed a 1.47 times lower tacrolimus oral clearance (CL/F) recovery with time compared with patients carrying at least one *CYP3A5*1* allele in their native intestine (Fukudo et al., 2008). In another study involving 51 Caucasian liver transplant recipients confirmed that tacrolimus dose requirements were dominantly influenced by the donors' *CYP3A5* gene polymorphisms. At 1, 3 and 6 months post transplantation, patients receiving a liver with at least one copy of *CYP3A5*1* allele had a significantly higher tacrolimus dose to reach the target trough concentrations compared to the patients receiving a liver homozygous for *CYP3A5*3* allele (Provenzani et al., 2011).

Table 3: Pharmacogenetic Studies of Tacrolimus in Kidney Transplant Recipients

Study reference	Recipient age	Study population	Patients Number	Posttransplant timeline	Major findings
MacPhee et al., 2004	adult	Black Caucasian South Asian Middle Eastern	178	2 Weeks	Patients with a *CYP3A5*1* allele had 2-fold lower dose-normalized tacrolimus concentrations than *CYP3A5*3/*3* homozygotes to achieve target blood concentrations
Hesselink et al., 2003	adult	Asian Black Caucasian	64	12 Months	*CYP3A5*1* allele carriers require higher tacrolimus dose to reach target trough concentrations than patients with the *CYP3A5*3/*3* genotype
Thervet et al., 2003	adult	Caucasian	80	1 Month	The weight-adjusted tacrolimus dose was significantly lower in patients with the *CYP3A5*3/*3* genotype than in those with the *CYP3A5*1/*1* genotype *CYP3A5*1* allele carriers showed a significantly lower C_0/D ratio compared to CYP3A5 non-expressers
Tsuchiya et al., 2004	adult	Japanese	30	28 Days	The dose-adjusted C_{max}, AUC_{0-12} and C_0 in *CYP3A5*1* carriers had a significantly lower value than the *CYP3A5*3/*3* carriers, though the daily dose of tacrolimus was significantly higher
Macphee et al., 2005	adult	Black Caucasian South Asian Middle Eastern	180	3 Months	CYP3A5 expressers require two fold higher doses of tacrolimus to achieve target blood concentration than non-expressers

Zhao et al., 2005	adult	Chinese	30	3, 6, 12 Months	Patients with the *CYP3A5*1/*3* required more tacrolimus to achieve the same target blood concentrations than those with the *CYP3A5*3/*3*
Tada et al., 2005	adult	Japanese	39	28 Day	Recipients who were *CYP3A5*1* carriers had a significantly lower tacrolimus dose-adjusted AUC_{0-12} and required a higher tacrolimus dose than *CYP3A5*3/*3* carriers
Zhang et al., 2005	adult	Chinese	118	1 Week; 1 , 3 Months	CYP3A5 expressers had a 2.2–4.3 times higher tacrolimus dose-adjusted C_0 than the CYP3A5 non-expressers. Patients with *CYP3A5*1*1* genotype had the lowest dose-adjusted C_0 compared to *CYP3A5*3/*3* carriers
Roy et al., 2006	adult	Asian Black Caucasian	44	3 Months	CYP3A5 expressers required a higher daily tacrolimus dose compared to non-expressers and achieved lower tacrolimus dose-adjusted trough blood concentrations compared to *CYP3A5*3/*3* allele carriers.
Ferraresso et al., 2007	paediatric	Caucasian	30	12 Months	Patients with *CYP3A5*1/*3* genotype required almost twice the daily dose of tacrolimus to achieve the target blood trough concentration and had lower tacrolimus trough blood concentrations than those with *CYP3A5 *3/*3* genotype
Quteineh et al., 2008	adult	Asian African Caucasian	136	1 Week; 1, 6, 12 Months	Patients possessing at least one *CYP3A5*1* allele (CYP3A5 expressers) needed significantly higher tacrolimus doses to achieve the same target trough concentration compared to patients with *CYP3A5*3/*3* genotype

Lopez-Montenegro Soria et al., 2010	adult	Spanish	35	6 Weeks	Patients with the heterozygote *CYP3A5*1/*3* genotype showed a lower tacrolimus concentration/dose ratios compared to the homozygote *CYP3A5*3/*3* genotype patients and required higher tacrolimus doses to achieve the target blood concentrations
Glowacki et al., 2011a	adult	African Caucasian	209	2 Weeks; 1, 3, 12 Months	Patients with at least one *CYP3A5*1* allele had significantly higher dose requirements and lower blood C_0 and C_0/daily dose ratio than patients homozygous for the *3 allele
Provenzani et al., 2011	adult	Caucasian	50	1, 3, 6 Months	The tacrolimus dose required to reach and maintain the desired trough concentrations was lower in the patients homozygous for *CYP3A5*3* allele compared to the heterozygous patients
Ferraris et al., 2011	paediatric	Caucasian	48	12 Months	Tacrolimus dose requirement was more than twofold higher in *CYP3A5*1/*3* than in *CYP3A5*3/*3* patients. *CYP3A5*3/*3* patients displayed higher C_0 and dose-normalized C_0 than *CYP3A5*1/*3* patients
Garcia-Roca et al., 2012	167 paediatric 124 adult	African Caucasian South Asian	291	6 Months	Both homozygous *CYP3A5*1/*1* and heterozygous *CYP3A5*1*3* expressers required higher tacrolimus doses than non-expressers *CYP3A5*3/*3*. No significant difference in tacrolimus dose was observed when comparing children with adults.
Chen et al., 2013	adult	Chinese	120	2 Week; 6, 18 Months	CYP3A5 expressers required significantly more tacrolimus than non-expressers to reach a comparable C_0 concentration. Although no significant difference in dose-adjusted C_0 , C_{max}, and $AUC_{0–12h}$ t was observed

Vannaprasaht et al., 2013	adult	Asian	68	6 Months	The induction dose of tacrolimus required for patients with *CYP3A5*1/*1* was 1.46- and 1.84-fold greater than that required in the *CYP3A5*1/*3* and in the *CYP3A5*3/*3* patients. The tacrolimus dose required for the maintenance phase was 1.3-fold higher than that in the *CYP3A5*1/*3* genotype and 2.4-fold higher than in the *CYP3A5*3/*3* genotype
Li et al., 2014	adult	Chinese	240	40 Days	Tacrolimus C_0/D of the patients with *CYP3A5*3/*3* was highest among the different *CYP3A5*1* allele genotype groups
Cusinato et al., 2014	adult	African Caucasian Amerindians	108	12 Months	The presence of the *CYP3A5*1* allele was strongly associated with lower dose-normalized concentration *CYP3A5*1* carriers and required a higher tacrolimus dose than CYP3A5 non-expressers
Lunde et al., 2014	adult	Caucasian	177	2, 7 Weeks	Heterozygous *CYP3A5*1* recipients had 42 % lower mean C_0/D ratio compared with homozygote carriers of *CYP3A5*3*
Kuypers et al., 2014	adult	Asian African Hispanic Caucasian	298	12 Months	Tacrolimus dose requirements were significantly higher in recipients carrying at least one *CYP3A5*1* allele as compared with homozygous *CYP3A5*3/*3* patients
Xing et al., 2015	adult	Chinese	96	1 Month	Patients carrying at least one the CYP3A5*1 allele showed a significantly lower C_0/D ratio compared to CYP3A5 non-expressers

CYP3A5 could be useful to predict the optimal tacrolimus dose prior to transplantation for rapid achievement of therapeutic tacrolimus concentrations. This was illustrated in a clinical trial by Thervet et al. (2008) who found that tacrolimus dosing based on the *CYP3A5* genotype allows earlier attainment of target blood concentrations. A randomized controlled study showed that kidney transplant recipients using tacrolimus doses according to the *CYP3A5* genotype reached the target C_0 significantly earlier than recipients used a standard regimen. Although more patients were within the desired tacrolimus target range early after transplantation, the improvement was modest with 29% of patients in target on the third day of tacrolimus dosing in the control group and 43% in the genetically adjusted group (Thervet et al., 2010). The study did not have sufficient statistical power to address clinically significant markers of efficacy failure and toxicity.

Twice daily tacrolimus, Prograf® (TD-Tac) and once a day tacrolimus (OD-Tac), Advagraf® have different dissolution properties. The prolonged release formulation of tacrolimus, Advagraf® is typically released further along the gastrointestinal tract. The immediate release formulation of tacrolimus, Prograf® is absorbed primarily in the jejunum and duodenum. The CYP3A protein expression may be higher in the jejunum than ileum. Consequently, CYP3A5 expression in the gastrointestinal tract may have less effect on the pharmacokinetics of OD-Tac than TD-Tac among both switching and de novo transplant patients (MacPhee, 2012).

Black subjects have a higher prevalence of CYP3A5 expression in comparison to individuals from other ethnic groups. In the Phase III study comparing Advagraf® to Prograf®, black subjects achieved approximately 70% of the trough blood concentration achieved by Caucasians for a given dose of both the Prograf® and Advagraf® preparations (Silva et al., 2007).

To date, few studies have focused on the impact of *CYP3A5* polymorphisms on the pharmacokinetics of conversion from twice- to once- daily tacrolimus formulations in renal transplant recipients. For the first time, Wehland et al. (2011) studied the pharmacogenetic effect of *CYP3A5*3* on two different tacrolimus formulations in a

cohort of 41 renal transplant patients over a period of 1 year before and after conversion. They reported that after conversion from Prograf® to Advagraf®, patients with *CYP3A5*3/*3* genotype required significantly lower tacrolimus doses to reach the target trough concentrations compared with patients with **1/*3* genotype in both formulations. Tacrolimus trough and dose-normalized trough concentrations of **3/*3* patients declined significantly after conversion to identical Advagraf® doses. However, they remained almost constant in **1/*3* patients. Conversely, a prospective single-centre study in stable kidney transplant recipients reported that the daily tacrolimus dose was significantly higher and the dose-adjusted $AUC_{0\text{-}24}$ was significantly lower in the CYP3A5 expressers for both Prograf® and Advagraf®. Additionally, the tacrolimus dose-adjusted $AUC_{0\text{-}24}$ and C_0 decreased significantly after the switch from TD-Tac to OD-Tac in CYP3A5 expressers (Glowacki et al., 2011b).

Again for the first time, Niioka et al. (2012) conducted a study in *de-novo* Japanese kidney transplant patients to investigate the pharmacokinetic and pharmacogenetic differences between once a day (OD-Tac) and twice-daily (TD-Tac) tacrolimus formulations. Twenty five patients received Tac-OD and 75 patients received TD-Tac. The authors found that despite no difference in the dose-adjusted $AUC_{0\text{-}24}$ and C_0 between the two formulations, CYP3A5 expressers had lower dose-adjusted $AUC_{0\text{-}24}$ and C_0 compared to non-expressers for each formulation. Among CYP3A5 expressers, but not non-expressers, the dose-adjusted $AUC_{0\text{-}24}$ and C_0 values were lower for OD-Tac than for TD-Tac. Another study in 34 Korean stable paediatric kidney transplant recipients showed that conversion from TD-Tac to OD-Tac did not affect the mean C_0 and $AUC_{0\text{–}24}$ in patients carrying *CYP3A5*1* allele but patients with *CYP3A5*3/*3* had a significantly lower C_0, C_{max} and $AUC_{0\text{–}24}$ after conversion (Min et al., 2013). Finally, a more recent study in paediatric kidney transplant recipients demonstrated that in CYP3A5 non-expressers dose normalized C_0 levels were higher compared to CYP3A5 expressers with TD-Tac, but not with OD-Tac (Lapeyraque et al., 2014). Further investigations into this controversial influence of *CYP3A5* polymorphisms during formulation switching are required.

3.2.1.2 CYP3A4

CYP3A4 is the most abundant cytochrome P450 expressed in the liver and the intestine. It accounts for approximately 60% of the total hepatic cytochrome P450 content. CYP3A4 is the most important drug-metabolizing cytochrome P450 enzyme in humans, it is involved in the metabolism of 45–60% of all currently used drugs (Danielson, 2002). CYP3A4 activity and protein content vary widely in the population, with a 10–100-fold between-individual variability. Most of the CYP3A4 polymorphisms have very low frequencies, and the phenotypic effects are weak and often controversial. Currently known genetic variants of CYP3A4 that change amino acids are rare and therefore can only explain a small portion of the observed variability (Elens et al., 2012). The most common CYP3A4 polymorphism is *CYP3A4*1B*. The frequency of *CYP3A4*1B* allele is 2%-10% of Caucasians, 4.2%-11% of Hispanics, 35%-67% of African-Americans, and about 0% of Asians (Chinese, Taiwanese and Japanese). The functional significance of this SNP is debatable and generally *in-vivo* studies are unable to prove an association between this polymorphism and the transformation of various drugs (Provenzani et al., 2013). Extrahepatic metabolism of tacrolimus by CYP3A4 in the gastrointestinal epithelium is responsible for the presystemic elimination of about half of the absorbed dose, whereas first-pass metabolism by CYP3A4 in the liver accounts for an additional 10% of elimination (Undre, 2003). Up to recently only one study has shown an association between the *CYP3A4*1B* allele and the required tacrolimus dose. It reported that dose-adjusted tacrolimus trough levels were lower in patients with the *CYP3A4*1B* allele than those with the *CYP3A4*1/*1* genotype (wild-type), however, this effect was not observed when the analysis was made only in the Caucasian population (Hesselink et al., 2003). A more recent study confirmed the relationship between *CYP3A4*1/*1B* genotype and tacrolimus dose-adjusted trough concentration. Among CYP3A5 non-expressers, a significant decrease in dose normalized tacrolimus concentrations was observed in *CYP3A4*1B* allele carriers compared to *CYP3A4*1*1* carriers at 6 months post-transplantation. However, in CYP3A5 expressers, the difference was not statistically significant. Therefore, *CYP3A4*1B* allele appears to be responsible for the decrease in dose normalized tacrolimus concentrations although the numbers were very small (Tavira et al., 2013).

The phenotypic significance of a recently identified SNP located in CYP3A4 intron 6 (rs35599367 C > T) is much more certain. The T-variant allele was linked both to reduced hepatic CYP3A4 mRNA expression and with decreased CYP3A4 enzymatic activity. This so-called *CYP3A4*22* allele also proved to have clinical implications, as it associated with the statin dose requirement for optimal plasma lipid control. mRNA levels in livers were 1.7-fold higher in *CYP3A4*22* wild-type CC; *CYP3A4*1/*1* than in *CYP3A4*22* (CT or TT) variant allele carriers (Wang et al., 2011). The *CYP3A4*22* allele is responsible for 7% of the variability in CYP3A4 mRNA expression (Elens et al., 2013b). This CYP3A4 SNP is relatively infrequent in Caucasians, 2.5%-6.9%. *CYP3A4*22* genotyping is classified into three groups CC, CT and TT genotypes that show high, moderate and low CYP3A4 expression activity, respectively (Elens et al., 2012, Wang et al., 2011).

A number of clinical studies have been designed to estimate the influence of this CYP3A4 SNP on tacrolimus clearance and dose requirement. An initial study by Elens et al. (2011b) revealed that the CYP3A4 rs35599367C>T polymorphism is correlated with a significantly altered tacrolimus metabolism. The dose-adjusted concentration in the intermediate metabolizer group was about 1.5- fold higher than the poor metabolizer group. While in extensive metabolizer group it was 4.4-fold higher for tacrolimus than the poor metabolizer group. When the *CYP3A4*22* and *CYP3A5*3* genotypes were combined, the results were more pronounced. In a second study, the same group demonstrated that early after transplantation, *CYP3A4*22* genotype is associated with tacrolimus metabolism alteration and lower tacrolimus dose requirements. T allele carriers require 33% lower tacrolimus doses than wild-type CC carriers to reach the target C_0 (Elens et al., 2011a). On the other hand, Tavira et al. (2013) found no significant differences in tacrolimus daily dose between the *CYP3A4*22* genotypes, either nominally or according to the *CYP3A5* genotype. Another study in 140 Brazilian renal transplant recipients, evaluated the impact of *CYP3A4* and *CYP3A5* SNPs and haematocrit on tacrolimus C_0/dose. There was no association between *CYP3A4*22* and tacrolimus dose requirement at 3 months after transplantation (Santoro et al., 2013). A study of paediatric heart transplant patients reported that *CYP3A4*22* SNP is linked to the changes in dose requirement of tacrolimus in the first 2 weeks following

transplantation. However, tacrolimus trough concentration (C_0) and C_0/dose ratio didn't change. CYP3A combined genotypes have a strong correlation with tacrolimus disposition. During the follow-up period, CYP3A poor metabolizers required approximately 20% and 50% less tacrolimus dose than intermediate and extensive metabolizers, respectively (Gijsen et al., 2013). Another study showed that steady-state oral clearance of tacrolimus was reduced in *CYP3A4*22* T-allele carriers compared with CC carriers, resulting in 50% lower tacrolimus daily dose requirements (de Jonge et al., 2014). It was confirmed that *CYP3A4*22* allele has a significant impact on tacrolimus pharmacokinetics by altering dose-adjusted blood concentrations of tacrolimus in renal transplant patients (Kurzawski et al., 2014). Pallet et al. (2015) in a brief communication reported that *CYP3A4*22* polymorphism is related to a slower metabolism of tacrolimus and a higher systemic exposure early after transplantation. Patients carrying *CYP3A4*22* variant may require 30% less tacrolimus dose than patients with the *CYP3A4 *1/*1* genotype. They also revealed that *CYP3A4*22* allele was predominantly found in CYP3A5 non-expressers and suggested that combining *CYP3A4* and *CYP3A5* genotypes might result in a better prediction of the optimal tacrolimus starting dose. However, a study by Lunde et al. (2014) found no association between *CYP3A4*22* polymorphism and tacrolimus C_0/D ratios. Furthermore, CYP3A combined allelic status did not give any additional information. Another study of 298 de-novo renal transplant recipient demonstrated that the dose requirements of tacrolimus were significantly lower in CYP3A5 non-expressers carrying at least one *CYP3A4*22* allele (slow metabolizers) compared with homozygous *CYP3A4*1/*1* CYP3A5 non-expressers (Kuypers et al., 2014). Moreover, Bruckmueller et al. (2014) reported that CYP3A extensive metabolizers (CYP3A5 expressers combined with *CYP3A4*1/*1*) required 2.1-fold higher doses than intermediate CYP3A metabolizers (*CYP3A5*3/*3* combined with *CYP3A4*1/*1*) or 2.7-fold higher doses than CYP3A poor metabolizers (*CYP3A5*3/*3* combined with *CYP3A4*1/*22 or *22/*22*).

*3.2.1.3 POR*28*

P450 oxidoreductase (POR) is essential for cytochrome P450 activity in humans. It transfers electrons from NADPH to microsomal cytochrome P450 enzymes, enabling

their activity and has been associated with increased *in vivo* CYP3A activity (de Jonge et al., 2011). POR mutations could alter the distribution of charge in the electron-donating domain, which might have quite different effects on the interaction of POR with different P450 enzymes (Flück et al., 2007). Consequently, POR might be a general limiting factor for drug metabolizing capacity. The *POR*28* (rs1057868C>T) mutation was the most common allelic variant on approximately 28% of all alleles. *POR*28* Genotype is classified into three allelic categories with high, moderate and low POR protein production, that are represented as TT, CT and CC, respectively (Zhang et al., 2013). *POR*28T* allele is also referred to as *POR*28* and *POR*28CC* allele is also known as *POR*1/*1* (Gijsen et al., 2014). *POR*28* SNP varies in frequency:19.1 % in African Americans, 26.4 % in Caucasian Americans, 36.7 % in Chinese Americans, and 31.0 % in Mexican Americans (de Jonge et al., 2011). *In vitro* studies showed that the *POR*28* C>T mutation affected CYP3A4 activity and the impact of this variant on catalysis by CYP3A4 was substrate-specific (Agrawal et al., 2010). Up to date, very few studies were carried out to explore the influence of genetic variations in the *POR* gene on tacrolimus metabolism. A study in healthy Chinese volunteers reported that no significant difference in tacrolimus pharmacokinetics was observed between *POR*28 CC* and *POR*28 CT/TT* genotypes. However, the *POR*28 CT* genotype had a significantly lower level of tacrolimus exposure (AUC, C_{max}) compared to the *POR*28 CC* genotype in CYP3A5-expressing subjects (Zhang et al., 2013). Another study in 298 adult renal transplant recipients showed that *POR*28T* allele carriers had significantly higher tacrolimus dosing requirements compared with *POR*28CC* homozygous patients. Additionally, the *POR*28* SNP is associated with significant increases in early tacrolimus dose-requirements in patients carrying a *CYP3A5*1* allele (CYP3A5 expressers) and did not affect tacrolimus trough blood concentrations and daily dose requirements in CYP3A5 non-expressers (de Jonge et al., 2011). A retrospective observational study in 52 heart transplant patients during the first year after transplantation found that the *POR*28* variant carriers had higher dose-adjusted trough concentrations with significant differences at 3 and 6 months after transplantation (Lesche et al., 2014). Another retrospective pilot study in 43 paediatric kidney transplant recipients in the first two weeks post-transplantation demonstrated that

CYP3A5 expressers carrying at least one *POR*28* allele had lower tacrolimus trough concentrations and lower concentration/dose ratios compared with CYP3A5 expressers with *POR*1/*1* genotype. In CYP3A5 non-expressers, tacrolimus disposition did not significantly change between POR genotypes. They also observed no significant differences in the tacrolimus dosing requirements between the POR genotype groups, neither in the CYP3A5 expressers nor in the CYP3A5 non-expressers (Gijsen et al., 2014). In a cohort of 298 *de-novo* renal transplant recipients, Kuypers et al. (2014) demonstrated that the CYP3A5 expressers with at least one variant *POR*28 T* allele (high metabolizers) had a significantly higher tacrolimus dose requirements than *POR*28 CC* homozygous patients, to maintain tacrolimus target C_0. This was from day 2 onward and continued throughout the first year after transplantation.

3.2.2 PXR

Pregnane X receptor (PXR), also known as steroid X receptor (SXR) or nuclear receptor subfamily 1, group I, member 2 (NR1I2) is a protein encoded by the *NR1I2* gene. It is highly expressed in the liver and the intestine. A variety of endogenous ligands such as steroids and bile acid salts, and exogenous ligands including drugs activate PXR, which alters their metabolism and regulates *CYP450* and the *ABCB1* gene expression (di Masi et al., 2009). The most frequent *PXR* polymorphisms are -25385C>T (rs 3814055) and 7635A > G (rs 6785049) single nucleotide polymorphisms. A study in 32 renal transplant recipients showed that *PXR* −25385C>T single nucleotide polymorphism was identified as a significant covariate for apparent oral clearance of tacrolimus. However, it was not clinically relevant in estimating the individual pharmacokinetic parameters of tacrolimus (Benkali et al., 2009). Moreover, Miura et al. (2008) reported that *PXR* 7635A>G SNP had no effect on tacrolimus pharmacokinetics, but was strongly associated with prednisolone pharmacokinetics. Conversely, a study in 31 *de-novo* renal transplant recipients reported an increase in tacrolimus clearance in subjects with the *PXR* 7635GG genotype (Press et al., 2009). An observational study in 35 renal transplant recipients found no effect of *PXR* −25385C>T polymorphisms on tacrolimus concentration/ dose ratio between heterozygote and homozygote genotypes (Lopez-Montenegro Soria et al., 2010). The clinical relevance of the influence of PXR receptor

genetic variants on tacrolimus metabolism is still unclear, especially immediately after transplantation where high doses of steroids are used.

3.2.3 ABCB1

P-glycoprotein (PGP) (1280 amino acids) is an efflux 'pump' that transports substances from the intracellular to the extracellular side of the cell membrane. It is a member of adenosine triphosphate (ATP)-binding cassette family of transport proteins (ABC transporters). The energy required for PGP to function is derived from its binding with ATP. The human gene encoding PGP is known as the *ABCB1*gene, previously known as *MDR1* (multidrug resistance protein 1). It is highly expressed in the apical membrane of enterocytes lining the gastrointestinal tract, renal proximal tubular cells, the canalicular membrane of hepatocytes and other important blood-tissue barriers such as those of the brain, testes and placenta (Flanagan *et al*., 2007). The most commonly studied *ABCB1* SNP is the 3435C>T polymorphism with a C to T substitution at position 3435 on exon 26 (rs1045642). According to P-gp expression, *ABCB1* genotyping is classified into three groups CC, CT and TT genotypes that show high, moderate and low P-gp expression activity, respectively (Li et al., 2006b). Consequently, The *ABCB1 CC* genotype is associated with a higher P-gp function compared with the CT and TT genotypes. The homozygous T-allele had more than 2-fold lower MDR-1 expression levels compared with homozygous CC genotype. The gastrointestinal absorption of P-gp substrates is inversely proportional to the P-gp expression level in the gut (Hoffmeyer et al., 2000). The variation in the oral bioavailability of tacrolimus is primarily caused by heterogeneity in the level of intestinal P-gp expression (MacPhee et al., 2002). However, this relationship is not confirmed in some studies (Tsuchiya et al., 2004, Haufroid et al., 2004). The frequency of *ABCB1* 3435 T allele occurs in 5% of African-Americans, 27% of Asians, 32% of Caucasians and 35% of Mexicans (Provenzani et al., 2013).

Some studies have reported a correlation between *ABCB1 3435* polymorphism and tacrolimus pharmacokinetics. Originally, MacPhee et al. (2002) in a study including 180 kidney transplant recipients found a significant association between the *ABCB1 3435*

polymorphisms and tacrolimus blood concentration at 3 months after transplantation. A study by Zheng et al. (2003) studied tacrolimus pharmacokinetics in relation to *ABCB1 3435* polymorphisms in 65 pediatric heart transplant patients and found a significant association between the *ABCB1* polymorphisms and tacrolimus dose, showing that *CC* recipients require a higher dose than the *CT/TT* patients at 6 and 12 months after heart transplantation (Zheng et al., 2003). Another study in 50 Chinese liver transplant patients investigating the relationship between *ABCB1* SNP and the tacrolimus daily dose and C/D ratio demonstrated that during the first month after transplantation, recipients carrying *ABCB1* T allele had a lower tacrolimus daily dose and higher C/D ratios than among *3435CC* recipients (Wei-lin et al., 2006). An observational study in 35 Spanish renal transplant recipients found a significant association between the *ABCB1* 3435 *T* allele and higher concentration/dose ratios of tacrolimus. CC genotype carriers showed up to 40% lower concentration/dose ratios compared with T variant allele carriers, suggesting that lower tacrolimus doses may be required for the T-allele carriers compared to C/C homozygotes (Lopez-Montenegro Soria et al., 2010). A study including 62 Han Chinese liver transplant recipients showed that Patients with *ABCB1C/C* homozygotes had significantly lower C/D ratios compared with *ABCB1* CT/TT variant carriers and required a little higher tacrolimus dose compared to those with *C/T* and *T/T* genotypes (Yu *et al*., 2011).

Most studies have failed to find any association between *ABCB1* genotype and tacrolimus pharmacokinetics. A study in 30 Japanese renal transplant recipients showed no significant difference in the tacrolimus dose, the dose-adjusted trough concentration, C_{max}, and $AUC_{0–12}$ between the *ABCB1CC* and *CT/TT* genotypes (Tsuchiya et al., 2004). In a study on 100 Caucasian renal transplant patients, Haufroid et al. (2004) found no significant association between tacrolimus dose-adjusted trough blood concentration or dose requirement and *ABCB1* genotype. Another study in 118 Chinese renal transplant patients reported no obvious correlation between *ABCB1 C3435T* polymorphisms and tacrolimus dose-adjusted C_0 from 1 week to 3 months after transplant (Zhang et al, 2005). Another study in Japanese renal transplant patients was carried out to investigate the influence of the *ABCB1 C3435T* mutation on tacrolimus pharmacokinetics between CYP3A5 expressers and non-expressers. They found no

association between the *ABCB1 C3435T* polymorphisms and tacrolimus pharmacokinetic parameter in either group (Tada et al., 2005). Moreover, Quteineh et al. (2008) in a study on 136 renal transplant recipients demonstrated that *ABCB1* polymorphisms did not have a significant association with tacrolimus daily dose and concentration/dose ratio. A Korean study in 321 livers and 185 kidney transplant patients demonstrated that *ABCB1* genotype had no correlation with tacrolimus concentration to adjusted-dose ratios in both liver and kidney transplant recipients (Jun et al., 2009). Gijsen et al. (2011) also demonstrated that, in 30 Canadian paediatric heart transplant patients, none of the *ABCB1 3435C>T* genotypes were associated with tacrolimus dosing requirements, tacrolimus trough concentrations, or concentration/dose ratio. This was supported by Provenzani et al. (2011) in a study on 51 liver and 50 kidney transplant Caucasian recipients and they found that during the first 6 months after transplantation, the *ABCB1 C3435>T* polymorphism had no effect on the dosage or the level / dose ratio. A retrospective study in 68 kidney transplant recipients also confirmed that no statistically significant correlation was noticed between the tacrolimus doses required for the induction and maintenance phases and *ABCB1* polymorphism (Vannaprasaht et al., 2013). A Chinese study in 216 liver transplant recipients confirmed these results and showed no obvious correlation of *ABCB1* polymorphisms with tacrolimus dose-adjusted C_0 and daily dose after transplant (Shi et al., 2013). Another retrospective study involving 90 paediatric *de novo* liver graft recipients also evidenced that the variants of *ABCB1* has minimal impact on tacrolimus disposition. They found no significant difference of the C/D ratios among the recipients with *ABCB1* polymorphisms (Chen et al., 2014). Further supporting these results, Lapeyraque et al. (2014) found no association between the concentration/dose ratio and *ABCB1* genotypes for either tacrolimus formulations (Prograf® and Advagraf®) in 19 stable paediatric renal transplant recipients.

Some other studies have reported the same outcome when combined between *ABCB1* variants and *CYP3A5* genotypes. MacPhee et al. (2002) reported that a minor association was found in the *ABCB1* allele variants on tacrolimus trough concentrations in CYP3A5 non-expressers. A Japanese study involving 39 kidney transplant recipients

showed that the *ABCB1 CC* and the *CT/TT* genotype subgroups had no significant differences in tacrolimus bioavailability in both *CYP3A5*1/*1+*1/*3* and *CYP3A5*3/*3* genotype groups (Tada et al., 2005). Furthermore, Loh et al. (2008), in a study on 18 Asian renal transplant recipients, found that upon evaluating the 2 genotypes for *CYP3A5* and *ABCB1* in combination, significant differences in tacrolimus Concentration/Dose ratios for the various groups reflected mainly the CYP3A5 polymorphism. A French study in 136 renal transplant recipients investigating the combined effect of *CYP3A5* and *ABCB1* polymorphisms on tacrolimus daily dose and concentration/dose ratio showed that the significant effect of *CYP3A5* on tacrolimus daily requirements was not modified by the allelic variants of *ABCB1* 3435C>T polymorphisms (Quteineh et al., 2008). Another study in 63 Chinese renal transplant recipients was made by Rong et al. (2010) to investigate the influence of the *ABCB1* 3435C>T polymorphism on tacrolimus pharmacokinetics between *CYP3A5*1* carriers vs *CYP3A5*3/*3* carriers. They concluded that no obvious influence on tacrolimus pharmacokinetics was observed between *ABCB1* CC and CT/TT genotype subgroups in both CYP3A5 expressers and non-expressers. In summary, a minor influence of the *ABCB1* 3435C>T genotype on tacrolimus pharmacokinetics has been identified in studies with sufficient statistical power that has not been detected in smaller studies.

3.3 Prednisolone Influence on Tacrolimus Pharmacokinetics

Corticosteroids are an important component of clinical immunosuppressive therapy during induction and maintenance of immunosuppression. Corticosteroids are also very effective when used at high doses to treat episodes of acute rejection. Prednisolone, a synthetic corticosteroid, is widely used immunosuppressive agent and is commonly used to treat and prevent acute rejection after organ transplantation. Prednisolone mechanism of action involves the inhibition of neutrophil, leukocyte and monocyte-macrophage accumulation at the site of inflammation. Prednisolone suppresses cell-mediated immunity through inhibiting genes that code for the cytokines, especially IL-2, which help T lymphocyte proliferation. Furthermore, prednisolone suppresses humoral immunity, causing B lymphocytes to express smaller amounts of IL-2 and IL-2 receptors. This diminishes both B lymphocyte clone expansion and antibody synthesis

(Taylor et al., 2005). Treatment with prednisolone for a long time is associated with well documented complications which include hypertension, diabetes mellitus, cataracts, osteoporosis, hyperlipidemia, weight gain and infections. Corticosteroid-avoidance or minimization is gaining popularity as the corticosteroid long term adverse effects can lead to transplant loss related to cardiovascular mortality and/or fatal infectious complications. The progressive improving potency of the other immunosuppressive drugs also helps to achieve corticosteroid-free immunosuppression. Although corticosteroid-free regimens have many advantages including lower rates of hypertension, diabetes mellitus and hyperlipidemia after transplantation, the rate of acute rejection remains lower in patients receiving corticosteroid-containing regimens (Abboudi and Macphee, 2012).

CYP3A and P-gp are involved in the metabolism of both tacrolimus and steroids. Steroids are a well-known inducer of both CYP3A and P-glycoprotein activity. In an *in-vitro* study investigating tacrolimus metabolism in liver microsomes prepared from normal rats and rats treated with dexamethasone showed that the rate of tacrolimus metabolism significantly increased in microsomes obtained from dexamethasone treated rats (Prasad et al., 1997). A study on animals showed that high dose steroid therapy in rats reduces tacrolimus blood concentrations due to the induction of CYP3A and P-gp in the liver and intestine (Shimada et al., 2002). Another study in renal transplant patients reported that the higher the steroid dosage used, the higher the tacrolimus dosage required to achieve target trough blood concentrations in these patients. The interaction also occurs even with low steroid dosage (Anglicheau et al., 2003a). Another study in kidney transplant recipients comparing the tacrolimus trough concentration and dose before and after prednisolone withdrawal found that withdrawal of steroids results in an increased systemic exposure to tacrolimus. The increase in systemic exposure to tacrolimus following 10 mg prednisolone withdrawal was higher (33-36%) than the increase after 5 mg prednisolone withdrawal (12-14%). This increase is only observed in AUC and is not associated with an increase in either C_{max} or t_{max} indicating that the processes of absorption remain unaffected so P-gp role seems less likely. It also indicates that the increase in tacrolimus exposure following steroid withdrawal is due to the reduction in the metabolic clearance as CYP3A4 induction by steroid wears off (van

Duijnhoven et al., 2003). Furthermore, Park et al. (2009) found an inverse correlation between prednisolone daily dose and tacrolimus exposures. Prednisolone dose reduction was associated with an increase in tacrolimus drug exposures. A recent study in 30 Serbian renal transplant patients demonstrated that during the first 6 months after transplantation, corticosteroid dose significantly influenced tacrolimus blood concentration. Corticosteroid tacrolimus interaction has more effect on male than female patients (Velickovic-Radovanovic et al., 2012).

3.4 Tacrolimus Within-Patient Variability

Tacrolimus is characterized by wide pharmacokinetics variation between individuals. To some extent, this variability is influenced by genetic and non-genetic factors and has been widely studied. In contrast to the between -patient variability, the within-patient variability is defined as fluctuating trough concentrations of a drug in a given period of time during which drug dosage was unchanged (Prytula et al., 2012).

Ekberg et al. (2009) demonstrated that approximately 50% of patients on low-dose tacrolimus (6 mg/day) were within the target range and this proportion increased over time during the first year after transplantation. Individual patients' analysis showed that only 11% of the patients were consistently within target range at all times during the first 2 months after transplantation. The within-patient coefficient of variation (CV) was 28% suggesting that the major reason for the difficulty to achieve and keep tacrolimus target concentrations is presumably the great within -individual variation of tacrolimus blood concentrations. Consequently, therapeutic drug monitoring (TDM) is used routinely to direct tacrolimus dosing within a defined therapeutic range of whole blood concentrations to achieve maximum efficiency and thus minimize the risk of sub-therapeutic or toxic blood concentrations which can lead to either rejection and graft loss or malignancy, serious infection and nephrotoxicity (Johnston and Holt, 1999). High within-patient variability complicates therapeutic drug monitoring as the drug concentrations will frequently be above or below the therapeutic window, putting the patient at risk for toxicity in the case of overexposure or for acute rejection in the case of underexposure (Prytula et al., 2012).

Borra et al. (2010) previously reported that high within-patient tacrolimus trough concentration variability was a risk factor long term chronic allograft nephropathy and graft loss in adult renal transplant recipients. Furthermore, an observational retrospective study performed in two Dutch pediatric nephrology centres reported that children and adolescents treated for late acute rejection have significantly higher within-patient variability in tacrolimus exposure than those who do not experience late acute rejection (Prytula et al., 2012).

Tacrolimus was initially introduced to the market as an immediate-release formulation, (Prograf®; Astellas Pharma Europe Ltd) that is administered twice a day. Recently, a prolonged -release formulation of tacrolimus (Advagraf®; Astellas Pharma Europe Ltd) has been developed to provide once-daily dosing with a similar efficacy and safety profile to twice-daily tacrolimus formulation, Advagraf®. Equivalent exposure is achieved at steady state with similar areas under the concentration/time curve (AUC), trough concentrations (C_0), and reduced peak concentrations (C_{max}). There is a good correlation between trough blood concentration and AUC allowing trough concentrations to be used for therapeutic drug monitoring, as is routine for twice-daily tacrolimus preparations (Alloway et al., 2005).

High within-patient variability in tacrolimus exposure is considered as a risk factor for allograft loss and late acute rejection. The reasons for this variability within individual patients are still unclear. Some factors, such time of tacrolimus dosing and interaction with food and medication noncompliance could be the reasons for this variability. The impact of food and the adherence rate seem to be significantly lower in Prograf® evening versus the morning dose. The presence of food in the gastrointestinal tract, particularly its fat content, significantly influences both the rate and extent of drug absorption. Bekersky et al. (2001b) demonstrated that food had a clinically significant influence in reducing tacrolimus relative bioavailability, as well as slowing its absorption. The effect on the rate of tacrolimus absorption was more pronounced in high-fat meals relative to low-fat meals. They also found that, taking tacrolimus one hour prior to a meal provides minor influence in tacrolimus relative bioavailability extent compared to taking tacrolimus in the fasting state. Additionally, the ingestion of

tacrolimus immediately after a meal or 1.5 hours subsequent to a meal had a more pronounced influence (Bekersky et al., 2001a). Accordingly, tacrolimus should be taken at least 1 hour before or 2 hours after eating and at the same time every day and any small deviations in the timing of drug intake may cause fluctuation in tacrolimus blood concentrations outside the therapeutic window with increased risk for episodes of acute rejection or toxicity.

Medication compliance remains a serious problem after transplantation. Non-adherence to medical treatment in transplant recipients is considered a major risk factor for graft rejection episodes and associated with graft loss. Even small deviations from the prescribed regimen (ie, <95% adherence) are associated with worse outcomes and significant financial implications (Laederach-Hofmann and Bunzel, 2000, Takemoto et al., 2007). Dosing complexity, including frequency of drug dosing and tablet or capsule burden for organ transplant patients may provide a motive for noncompliance to the therapeutic regimen (O'Grady et al., 2010). Claxton et al. (2001) confirmed that the prescribed number of doses per day is inversely related to compliance and reported that simpler, less frequent dosing regimens resulted in better compliance across a variety of therapeutic classes. In this situation drugs providing once a day administration can improve adherence ensuring appropriate drug blood concentration. Ichimaru et al. (2008) performed a study to survey the actual treatment adherence status of patients to Prograf® that need to be taken twice daily (morning and evening). They found that the adherence rate decreased over time following transplantation and this was more pronounced with the evening doses. Weng et al. (2005) demonstrated a significant association between dosing frequency (once-daily vs twice-daily) and adherence among adult transplant recipients.

A change from Prograf® to Advagraf® reduces 'pill-burden', simplifies the once daily regimen and results in only having to coordinate one meal per day around drug administration. The recommended dose for conversion from Prograf to Advagraf is considered to be on 1:1 mg basis. Simpler dosing regimens have been shown to facilitate better adherence performance. Doesch et al. (2010) demonstrated a significant improvement in heart transplant patient adherence after a switch to modified-release

tacrolimus, Advagraf® which was generally safe and well tolerated. Another study in kidney transplant recipient found that compliance was improved among patients treated with once-daily tacrolimus compared with twice-daily tacrolimus (Alloway et al., 2007). A more recent study reported an improvement in patients' adherence after switch to OD-Tac due to lack of the evening dose associated with a higher rate of missed doses than the morning dose (Kuypers et al., 2013).

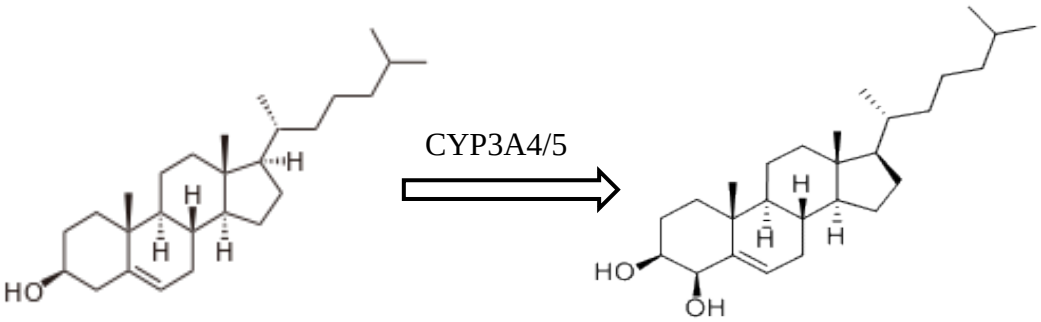
In contrast to between-patient variability, only few studies have investigated the differences in within-patient variability (WPV) when Prograf® was changed to Advagraf®. A study by Wehland et al. (2011) investigating the impact of Prograf® to Advagraf® conversion on 41 stable Caucasian renal transplant recipients showed that both drugs exhibited similar within-patient variability of dose-normalized trough levels. However, a retrospective, single-centre, polish study in 52 renal transplant patients examined the impact of switching from Prograf to Advagraf on daily drug dose and blood concentration variability, showed that conversion from twice daily to once-daily tacrolimus late after transplantation ensure greater stability of drug blood concentrations compared to the standard tacrolimus form (Kurnatowska et al., 2011). Another prospective, single-centre study was performed to investigate the change of within-patient variability in 129 stable Taiwanese kidney transplant recipients after conversion from twice-daily Prograf® to the same daily dose of once-daily Advagraf®. They found that conversion from Prograf to Advagraf® is associated with a significantly lower within-patient variability of tacrolimus C_0 (Wu et al., 2011). Additionally, a multi-centre, prospective study performed in stable adult kidney transplant recipients showed that between- and within-subject variability in systemic exposure to tacrolimus (%CV for $AUC_{0–24}$) were similar for both once and twice a day tacrolimus (van Hooff et al., 2012). Recently, Shuker et al. (2014) studied two hundred and forty seven ethnically different stable renal transplant recipients and found that conversion from once to twice a day tacrolimus significantly reduces tacrolimus exposure and it does not reduce WPV in tacrolimus exposure. However, another recent mixed race study showed a significant reduction in within-patient variability on conversion to once daily, modified-release

tacrolimus in a subgroup of 58 liver transplant recipients in the late conversion cohort (Considine et al., 2015).

Another explanation for this within-patient variability could be an individual's cytochrome P450 3A (CYP3A) genotype. Very few studies have focused on the relation between within-patient variability and genetic polymorphism, particularly *CYP3A5* polymorphism in twice daily tacrolimus formulation. Recently, a bioequivalence study in healthy Korean volunteers suggested that not only between-individual variability, but also WPV is correlated with the *CYP3A5* genotype (Yong Chung et al., 2010). In a single-centre retrospective study involving 249 Korean renal transplant patients concluded that within-individual variability in tacrolimus clearance is not related to *CYP3A5* genotype and suggested a strong association between WPV of tacrolimus trough concentrations and the development of acute rejection (Ro et al., 2012). It has been recently reported that a significant reduction in within-patient variability (%CV) of tacrolimus C_0 on conversion to once-daily tacrolimus among Taiwanese kidney transplant recipients. They also found that the %CV of tacrolimus C_0 decreased significantly in patients with at least *CYP3A5*1* allele and that there was no difference in tacrolimus trough concentration %CV between CYP3A5 expressers and non-expressers in both Prograf and Advagraf® (Wu et al., 2014). However, another study of non-Asian transplant patients with mixed ethnic background reported that within-patient variability of tacrolimus clearance is not associated with *CYP3A5* genotype (Pashaee et al., 2011). These results were recently supported by Spierings et al. (2013) in a study of 118 ethnically different renal transplant recipients, which concluded that within-patient variability of tacrolimus clearance was not associated with *CYP3A5* genotype in stable renal transplant recipients. Further research is required to provide more clear vision into what are the causes of this within-patient variability of tacrolimus clearance in patients treated with different tacrolimus formulations.

3.5 4β-hydroxycholesterol as a Potential Biomarker for CYP3A5 Activity

Phenotyping of CYP3A catalytic activity, determines to a large extent the substantial between-individual variation with respect to drug metabolic clearance and consequently, their bioavailability, elimination, and interaction with other drugs. Thus, having a marker for this activity seems to be of special importance for proper dose adjustments and induction potential screening of new drugs (Suzuki et al., 2013a). Furthermore, CYP3A enzymes are responsible for the metabolism of endogenous compounds such as cholesterol, steroid hormones (e.g., testosterone), bile acids and vitamin D (van Waterschoot and Schinkel, 2011). 4β-hydroxycholesterol (4β-OHC) is found in the human circulation and is produced from the enzymatic conversion of cholesterol by members of the P450 3A subfamily (**Figure 14**).



Cholesterol **4β-hydroxycholesterol**

Figure 14: Structure of 4β-hydroxycholesterol

Several studies showed that patients treated with CYP3A4 inducers had a marked increase in 4β-hydroxycholesterol concentrations, while patients treated with CYP3A4 inhibitors were shown to have markedly decreased levels of 4β-hydroxycholesterol (Josephson et al., 2008, Goodenough et al., 2011, Diczfalusy et al., 2009, Lutjohann et al., 2009). A study by Diczfalusy et al. (2008) found that the concentration of 4β-OHC increased with the number of active *CYP3A5*1* alleles. They suggested that 4β-OHC is not only formed by CYP3A4, but also by CYP3A5. Recently, 4β-hydroxycholesterol (4β-OHC) has been shown to be an endogenous marker of P450 3A activity in clinical

practice (Diczfalusy et al., 2011). Moreover, it has been reported that 4β-OHC concentration is associated with cholesterol plasma concentration which might be affected by some drugs. Therefore, 4β-OHC: cholesterol ratio may be more suitable to use as an alternative to 4β-OHC itself as a CYP3A measure (Diczfalusy et al., 2011).

The immunosuppressive drug, tacrolimus has a narrow therapeutic index and its pharmacokinetics shows wide between- and within-individual variability (Amundsen et al., 2012). CYP3A5 has been identified as the major enzyme responsible for tacrolimus metabolism. It has been reported that *CYP3A5* polymorphisms have a remarkable influence on tacrolimus trough blood concentration and dose requirements in stable kidney transplant patients (Glowacki et al., 2011a, Shi et al., 2013, Hesselink et al., 2003). Estimation of CYP3A activity before transplantation or starting tacrolimus treatment may help to prevent the possibility of rejection or toxicity. Moreover, 4β-OHC has been reported to be a potent and useful endogenous biomarker for CYP3A activity especially in kidney transplant patients. Therefore, 4β-OHC may be a useful biomarker for prediction of tacrolimus dosing in renal transplant patients.

Chapter 4. Comparative Pharmacokinetics Assessments of Immediate- and Prolonged-Release Tacrolimus: Associations with *CYP3A5* and *ABCB1* Genotypes.

4.1 Objective of the Study:

1. To compare the pharmacokinetics of tacrolimus, measured by a validated LC/MSMS method, using two tacrolimus formulations: immediate-release Prograf® or Adoport® and prolonged-release Advagraf®.
2. To study the Associations between *CYP3A5*3* and *ABCB1* 3435C>T SNPs and the pharmacokinetics of these formulations within patients.

4.2 Materials & Methods

This study was sponsored and monitored by the Joint Research and Enterprise Office, St. George's University of London and it has been supported by an unrestricted research grant from Astellas Pharma Ltd. The study protocol was approved by National Research Ethics Service Committee (**REC Number: 09/H0707/91**). The study is an open-label pharmacokinetic study with a crossover design.

4.2.1 Eligibility Criteria

4.2.1.1 Inclusion Criteria

1) Renal transplant recipient at least 6 weeks after transplantation on treatment with twice daily tacrolimus with planned change in treatment to once daily Advagraf® (prolonged release tacrolimus) as part of standard care.
2) Aged at least 18 years.
3) Treatment with no more than 5 mg prednisolone daily.
4) Signed and dated informed consent obtained before screening and before the performance of any protocol-specific tests.

4.2.1.2 Exclusion Criteria

1) Unstable renal transplant patients.
2) Aged less than 18 years old.
3) Treatment with more than 5 mg prednisolone daily.
4) Treatment with potent cytochrome P4503A and P-glycoprotein inducers (such as carbamazepine, phenytoin, and rifampicin) inhibitors (such as diltiazem, erythromycin, fluconazole, and verapamil) or any less commonly prescribed potent inducer or inhibitor.
5) Development of intolerance to tacrolimus preparations.
6) Inability to obtain satisfactory venous access.
7) In the event of an adverse event where study continuation is considered to be inappropriate by the investigator.
8) Withdrawal of consent.

4.2.2 Study Design

Stable adult renal transplant patients in follow-up at three sites of the local transplant network (St. George's, St. Helier and Brighton Hospitals) were invited to participate in the study. At the time of enrolment, patients were informed of the purpose, duration and risks of the study, and they were requested to sign a written informed consent form (**Appendix 1**). After signing the consent form, patients were checked for their eligibility to participate in the study based on the inclusion and exclusion criteria. Eligible subjects were genotyped using DNA prepared from peripheral blood samples collected at a time when undergoing venepuncture as part of routine care as shown in **Figure 15**. The evaluations and tests performed also included:

- Taking patient demographic data, including (Weight, Sex, Ethnic group, Age and date of birth, Original renal disease)
- Recording any significant past medical history
- Carrying out a physical examination
- Checking vital signs (including Pulse rate, Blood pressure)
- Recording all concurrent medications

- Recording full blood count from most recent outpatient clinic visit
- Recording Biochemical series (including urea, creatinine, electrolytes, calcium, alkaline phosphatase, AST) from most recent outpatient clinic visit.

Patients were informed about the possibility of withdrawing from the study at any time. They were requested to report any abnormality occurring throughout and after the study. The results of clinical evaluations and any abnormalities were documented in individual case report forms (**Appendix 2).**

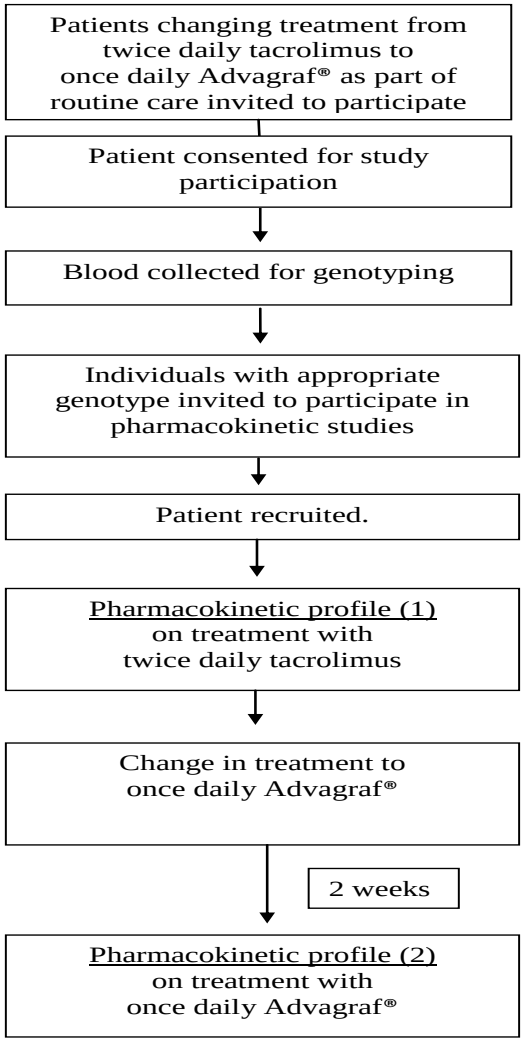


Figure 15: Study Flow Chart of Twice-Daily Tacrolimus and Advagraf® Pharmacokinetic Profiles using Crossover Design.

4.2.3 Study Treatments

This study measured drug concentrations for patients' standard therapy. During twice daily tacrolimus treatment, tacrolimus dose is usually adjusted to achieve 12 hour post-dose whole blood concentrations of 8-12 µg/L up until three months after transplantation and thereafter 5-8 µg/L. When the treatment was changed from twice daily tacrolimus to Advagraf® the same total daily dose was administered and adjusted to maintain trough blood concentrations within the target range. In order to standardise prednisolone CYP3A inducing effect, only patients treated with no more than 5 mg prednisolone daily were recruited.

4.2.4 Genotyping Determination

DNA was extracted from peripheral blood samples using QIAamp DNA Blood Mini Kit (QIAGEN®, West Sussex, UK). *CYP3A5*1/*3* and *ABCB1 3435C>T* polymorphisms were genotyped using real time polymerase chain reaction (PCR), a LightCycler® based technique. The samples were amplified using specific primer sequences.

4.2.4.1 Materials and Methods

QIAamp DNA Blood Mini Kit was obtained from QIAGEN® (West Sussex, UK). The LightCycler FastStart DNA Master Hybridisation Probe kit was supplied from Roche® (West Sussex, UK). Custom-made primers and probes were purchased from Tib Molbiol® (Berlin, Germany). PCR was performed using LightCycler® 1.0 supplied from Roche® (Lewes, UK). Ethanol was supplied by VWR. Deionised water was prepared on site (Millipore).

4.2.4.2 DNA Extraction and Purification Procedure

Ethylenediaminetetraacetic acid anticoagulated whole blood samples were collected and stored at –20°C until DNA isolation. QIAamp DNA Blood Mini Kits were used to extract and purify DNA from 200 µl of stored EDTA anticoagulated whole blood according to the manufacturer instructions. The extracted DNA samples were stored at –20°C until analysis.

*4.2.4.3 CYP3A5*3 and ABCB1 3435C>T Genotyping*

*4.2.4.3.1 CYP3A5*3 Genotyping*

FastStart DNA master Hybridization Probe kits was used for DNA amplification using two specific primers, forward and reverse primer. The forward primer sequence was ACTGCCCTTGCAGCATTTAG and the reverse primer sequence was CATACCCCTAGTTGTACGAC. Two hybridization probes were used, the anchor and sensor probes. The sequence of the Anchor probe was 5′-LCRed640-GTTTGGACCACATTACCCTTCATC-3′- Phosphate and the sensor probe sequence was 5′-CTTTTGTCTTTCAATATCTCTTCCC-3′-fluorescein. The reaction mixture made up of 0.5 µM of each primer and 0.3 µM of the probe. The final Mg^{2+} concentration in the reaction mixture was adjusted to 3 mM. 2 µL of DNA samples were loaded into composite glass capillaries (Roche Molecular Biochemicals) containing 18µL of the reaction mixture, centrifuged, and placed in the LightCycler sample carousel. Three control samples were included in each LightCycler run, two positive controls and one negative control (Deionized water).

The programme was designed as follows: pre- incubation phase at 95°C for 10 min to activate the Taq polymerase enzyme, followed by amplification phase, which includes 45 cycles of denaturation (95°C, 10s), annealing (55°C, 5 s) and extension (72°C, 15 s). Then this was followed by melting curve analysis stage, which involved annealing at 40°C for 50s, followed by an increase in temperature at a rate of 0.1°C/s, with continual fluorescence detection up to 95°C. F2 mode was used for fluorescence display. Light Cycler Software was used to convert melting curves into melting peaks by plotting the negative derivatives of fluorescence against temperature (-dF/dT) (Fredericks et al., 2005).

4.2.4.3.2 ABCB1 3435C>T Genotyping

FastStart DNA master Hybridization Probe kits were used for DNA amplification using two specific primers, forward and reverse primer. The forward primer sequence was TGTTTTCAGCTGCTTGATGG and the sequence of the reverse primer was

AAGGCATGTATGTTGGCCTC. Two hybridization probes were used. The sensor probe sequence was LC640-GGAAGAGATCGTGAGGGCAG--PH. The 3′-fluorescein-labeled anchor probe sequence was GACAACAGCCGGGTGGTGTCA--FL. PCR was performed in a reaction volume of 20μL with 0.2 μmoL each primer, 0.2 μmoL anchor and sensor probes, and 2μL of genomic DNA. The final Mg^{2+} concentration in the reaction mixture was adjusted to 3 mM. The samples were loaded into composite glass capillaries (Roche Molecular Biochemicals), centrifuged, and placed in the LightCycler sample carousel. After an initial denaturation step at 95°C for 10.0 min, amplification was performed using 45 cycles of denaturation (95°C for 5 s), annealing (55°C for 10 s), and extension (72°C for 20 s). After amplification was complete, a final melting curve was recorded by annealing at 40°C for 20s, followed by an increase in temperature at a rate of 0.1°C/s, with continual fluorescence detection up to 85°C. F2 mode was used for fluorescence display. Light Cycler Software was used to convert melting curves into melting peaks by plotting the negative derivatives of fluorescence against temperature (-dF/dT) (Nauck et al., 2000).

4.2.5 Determination of Ethnicity

Patients were classified by ethnicity based on the patient's transplant assessment records or their self-report as follows:

- White: any Caucasians, white British and any other white background
- Black: any ancestry from sub-saharan Africa or any other black background including Caribbean
- Asian: ancestry from India and any other south Asian background, not including any East Asians (Koreans, Chinese and Japanese).

4.2.6 Study Procedure

Subjects were divided into four groups according to their genotype, as shown in **Table 4**. The target size for each group was 30 patients. The sample size required was calculated using an anticipated CV of 20% and a power of 80%, within each of the four cohorts individually. From previous data, tacrolimus systemic exposure (AUC_{0-24}) for

Prograf® was slightly higher than that for Advagraf® [ratio 1.10]. In order that the 90% confidence interval for the treatment ratio lies entirely within 0.8 – 1.25, the total number of subjects required in each group was 30. The sample size calculation was performed using nQuery Advisor 5.0 [table MTE3-1].

Table 4: Study Genotype Groups of *CYP3A5* and *ABCB1* Alleles.

***CYP3A5* genotype**		***ABCB1* genotype**	**Number of patients**
**1/*1* or **1/*3*	CYP3A5 expressers	*CC*	30
**1/*1* or **1/*3*		*CT/TT*	30
**3/*3*	CYP3A5 non-expressers	*CC*	30
**3/*3*		*CT/TT*	30
Total			120

Patients involved in the study adopted a dosing schedule for one week prior to the study where the current dose of twice daily tacrolimus was administered twice daily at 08:00 and 20:00. An initial pharmacokinetic profile of the twice daily dose tacrolimus was measured. Treatment was then changed to the same daily dose of Advagraf®, which was administered once daily at 08:00 and then two weeks later, a further pharmacokinetic profile was measured after a single morning dose of Advagraf®, as shown **Figure 16**.

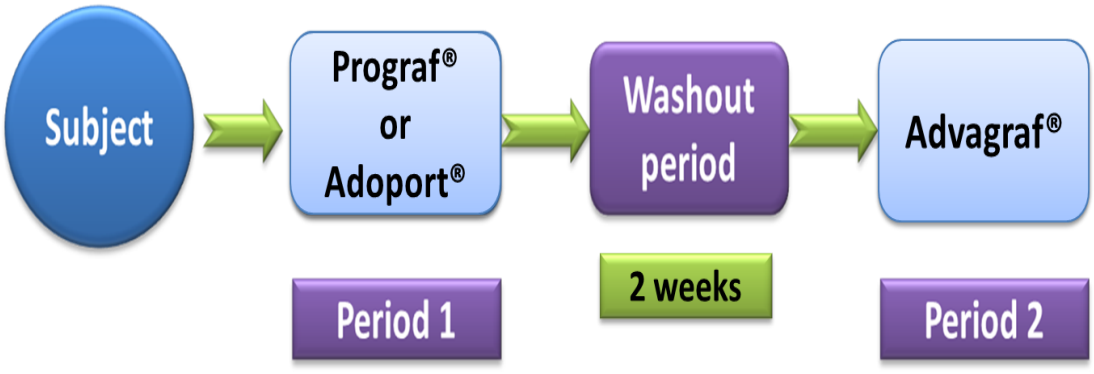


Figure 16: Twice-Daily Tacrolimus – Advagraf® Study Design.

A series of blood samples were collected at 0, 0.5, 1, 2, 4, 6, 8, 12, 12.5, 13, 14, 16 and 24 hours post-dose for twice daily tacrolimus and at 0, 1, 2, 3, 4, 6, 8, 10, 12 and 24 hours post-dose for Advagraf® as shown in **Figure 17**. This required a 24 hour stay in the hospital for the first set of samples with the option to go home between the 12 hour and 24 hour samples on the second sampling day. Subjects were required to fast for 2 hours before taking the drug dose and for 1 hour afterwards. A venous cannula was placed into the hand or arm for blood sampling and was kept in place throughout the sampling period. Five mL of blood was collected at each time point into EDTA tubes. Blood samples were stored frozen at approximately -20ºC until the drug bioanalysis.

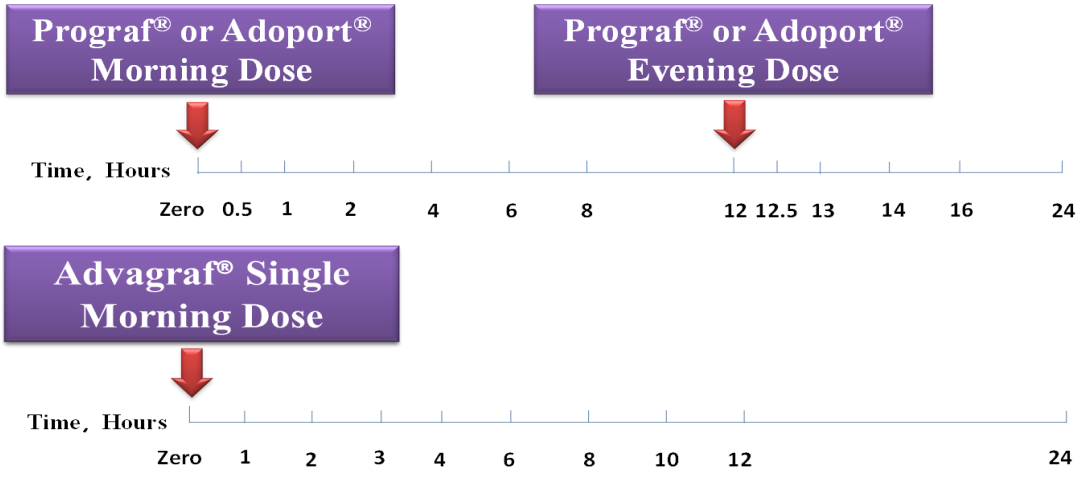


Figure 17: Blood Sampling Schedule Following Administration of Twice-Daily Tacrolimus Formulations and Advagraf®.

4.2.6.1 Tacrolimus Analysis

This analysis procedure was conducted following the method previously validated according to FDA guidelines at Analytical Services International Ltd, St George's - University of London titled "The Validation of an LC-MS/MS Assay to Measure Tacrolimus and Everolimus in Human Blood". It allows the analysis of tacrolimus in whole blood at concentrations ranging from 1-50 µg/L with a correlation coefficient between concentration and peak area ratio (r) of 0.998. The lower limit of quantitation was 0.25µg/L. Both the intra-day and inter-day accuracy values were all within 93.1-109.4% and 95.1-105.3%, respectively. In addition, the average of absolute recovery values was 80.8%.

4.2.6.1.1 Chemicals and Reagents

Tacrolimus was supplied by Enzo Life Sciences and ascomycin (IS) was supplied by Sigma-Aldrich, together with Zinc sulfate heptahydrate. HPLC-grade methanol and Methyl-tert-butyl ether (MTBE) were obtained from Rathburn Chemicals Limited. Acetonitrile (HPLC grade) was supplied by VWR. Ammonium acetate, Sodium hydroxide, ethanol and ethanediol, were purchased from VWR. EDTA anticoagulated tacrolimus -free human whole blood was supplied from Biological Specialty Corp, Colmar, Pennsylvania, USA. Deionised water was prepared on site (Millipore).

4.2.6.1.2 Stock Solutions

Calibrators and control samples were prepared in EDTA-anticoagulated whole blood, using separate stock solutions prepared in methanol. Six non-zero calibrators (nominal values of 1.0, 2.5, 5.0, 10.0, 25.0, and 50.0 µg/L) and three control samples (nominal values of 3.0, 15.0, and 30.0 µg/L) were prepared. Calibrators and controls were aliquoted and stored at approximately 20°C before use. A stock solution of ascomycin (internal standard) was prepared in ethanol-ethanediol-water (50:25:25) to give a concentration of 50 µg/L.

4.2.6.1.3 Extraction Procedure

For sample preparation, 50µL of calibrators, quality control, or patient samples; 50µL of internal standard, 1 mL of precipitating solution (500 mL zinc sulphate solution (25 g/L); 200 mL of acetonitrile; and 300 mL methanol) were pipetted into 2-mL Eppendorf vials. The vials were mixed for 5 min and then centrifuged at; 13,200 rpm for 5 min using Eppendorf 5415D Centrifuge (Eppendorf, England, UK). The supernatant was decanted into 4.5-mL polypropylene tubes with 100 µL of 0.1 mol/L sodium hydroxide and 2 mL of MTBE. After mixing for 5 min, the tubes were centrifuged at 3000rpm for 5 min. The organic solvent was then transferred to 4.5-mL conical polypropylene tubes, placed in a Savant SpeedVac Sc200 (Thermo Life Sciences) at 60°C, and evaporated to dryness. The dried extracts were reconstituted with 250 µL of 80% methanol, mixed for 5 min, and then transferred to autosampler vials, which were placed in an autosampler

tray set at ambient temperature. The autosampler injected a 10µL aliquot of each extract onto the analytical column (**Figure 18**).

4.2.6.1.4 Chromatographic and Mass Spectrometric conditions:

Validated LC-MS/MS method was used for the analysis of tacrolimus in the whole blood. The instrument used composed of Agilent HPLC comprises Pump, auto-sampler (Model: 1100 series) and auto injector coupled with Triple quadrupole AB Sciex API-4000 LC-MS/MS system. The reverse phase Alltech Alltima C18, 5µm, 150×2.1 mm column (Merck (BDH) Limited, Poole, Dorset, England) was used for the separation of tacrolimus and internal standard at temperature of 65°C using an Agilent series 1100 column oven. The mobile phase was pumped isocratically at a flow rate of 0.4 mL/min and consisted of a mixture of mobile phase A (Methanol) and mobile phase B (De-ionized water + 5mL/L 2M ammonium acetate) at a ratio of 82:18 respectively. The retention times were 2.9 for tacrolimus and 2.8 for ascomycin and the total run time was 4 min. The sample injection volume was 10 µL. Tandem mass spectrometric detection and quantification was performed in the positive electrospray ionization mode using multiple reaction monitoring (MRM) mode. The transitions selected were m/z 821.5/768.5 for tacrolimus and m/z 809.5/756.5 for ascomycin. Nitrogen was used as the collision gas. The NM20ZA high purity nitrogen and air generators were supplied by Peak Scientific Instruments, Scotland. Gas settings in ml/min were: Collision gas: 8, curtain gas: 10, ion source gas 1: 35, ion source gas 2: 30. Ion spray voltage: 5500 V, and temperature: 450°C. Entrance potential (EP), collision energy (CE), and collision cell exit potential (CXP) in voltage were 10, 29 and 18 V, respectively for all compounds. The dwell times were set at 300 msec. Declustering Potential (DP) was 81 for tacrolimus and 76 for ascomycin. A Windows PC running Analyst 1.3.2 software was used to control the LC-MS/MS, record the output from the detector, perform integration of peak areas and calculate the tacrolimus concentrations. The Analyst software was supplied by Applied Biosystems, England (**Figure 18**).

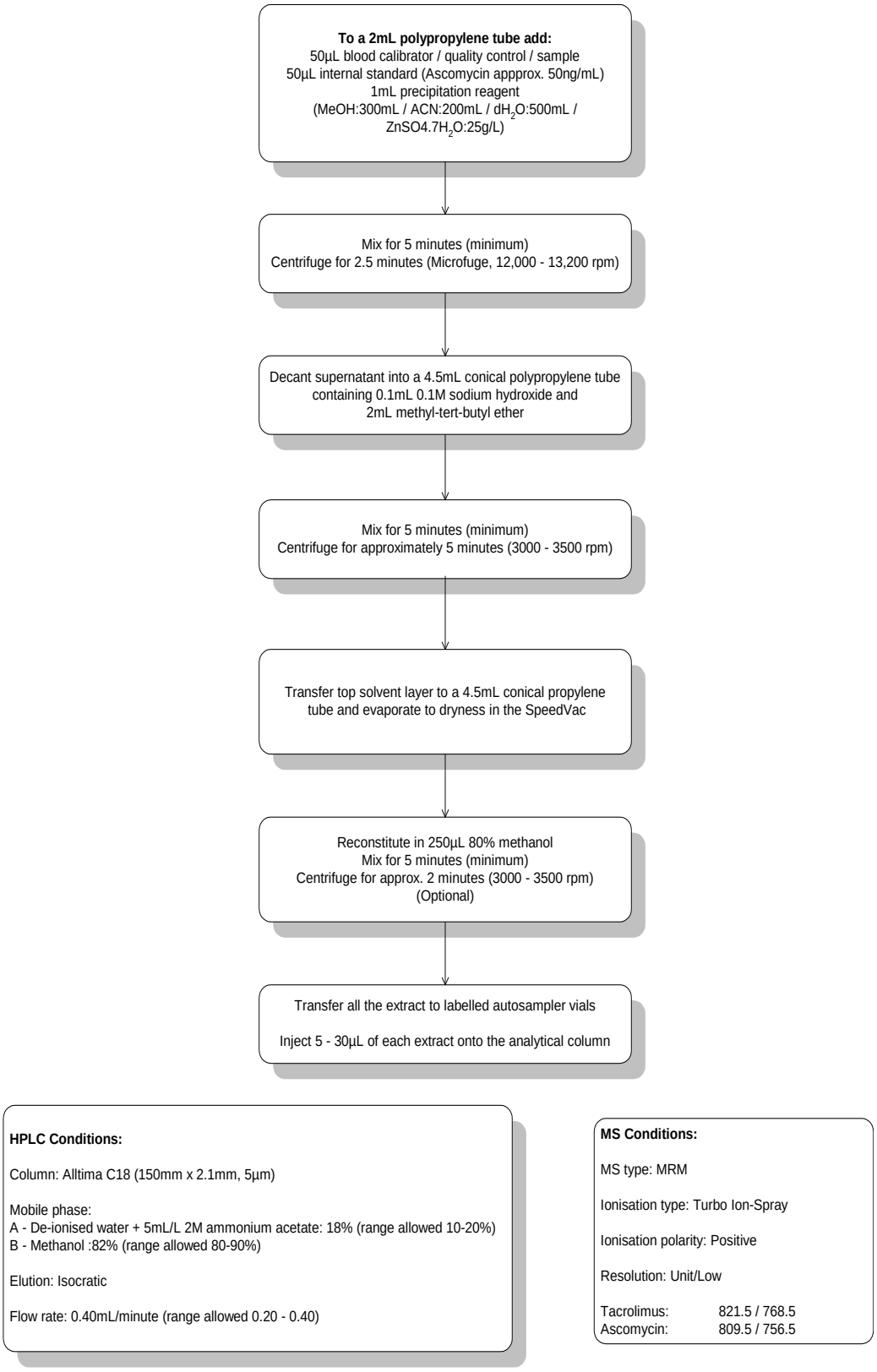


Figure 18: Schematic Diagram of the Tacrolimus Extraction Procedure.

4.2.7 Statistical and Pharmacokinetic Analysis

Statistical analysis was performed using Minitab statistical software (Minitab 17). According to the FDA and EMA guidelines patients' data were log transformed (FDA., 2003, EMA, 2010a). Then the log-transformed data were analysed using analysis of variance (ANOVA) with factors for genotype group and treatment. 90% confidence intervals around the ratio in means for twice-daily tacrolimus Prograf® and Adoport®: Advagraf® within each genotyping group was compared to the FDA bioequivalence margin of 0.8-1.25 where the differences in systemic drug exposure up to 20% are not clinically significant (FDA., 2003). EMA guidelines tightened the bioequivalence margin for tacrolimus AUC to 0.9-1.11, where the differences in systemic drug exposure up to 10% are not clinically significant (EMA, 2010a). See Section 2.2.1 page 63. The area under the plasma concentration-time curve (AUC) was calculated using the linear trapezoidal method. The peak plasma concentration (C_{max}) and t_{max} (the time to achieve C_{max}) were obtained from direct visual inspection of plasma concentration versus time curves. The allele and genotype frequencies of the *CYP3A5*3* and *ABCB1 3435C>T* polymorphisms were assessed for deviation from the Hardy–Weinberg equilibrium using the chi-square test.

4.3 Results

4.3.1 Pharmacokinetics and Bioequivalence of Tacrolimus Preparations in Stable Renal Transplant Patients.

The study was designed to compare the standard practice tacrolimus (Prograf®) versus Advagraf®. On the way through the study, the renal unit changed their standard practice tacrolimus to Adoport®. So we started with Prograf® and then we had to continue with Adoport®. Based on FDA guidelines and EMA guidelines both formulations were considered bioequivalent (Alloway et al., 2012, MEB, 2012). This had been checked in our study cohort and we confirmed that both formulations were bioequivalent according to FDA guidelines but not EMA criteria and therefore all patients treated with both formulations were combined in one group, twice daily tacrolimus group.

4.3.1.1 Patient Population

Of 75 stable kidney transplant patients who were screened and considered eligible for participation, 11 withdrew before the study began. Therefore, 64 patients (43 men, 21 women; 39 White, 12 Black, 13 Asian; mean [SD] age, 55 [13] years; age range, 21-78 years; mean weight, 76.4 [15.2] kg; mean height, 170.4 [8.6] cm) was recruited, and all participants completed both study periods. The mean (SD) time post-transplant was 4.1 (4.6) years (median 1.8 years, range 0.3–22.8). 34 patients had received a graft from a deceased donor (59.3%). Of these 64 patients, 25% had diabetes mellitus, and 61% were receiving maintenance steroids. 19 patients (29.7%) were receiving mycophenolate and 14 were receiving Azathioprine (21.9%) at baseline and throughout the study. Forty-eight patients (75%) were receiving with Prograf® and 16 patients (25%) were receiving Adoport®. The demographic characteristics and immunosuppression therapy are shown in **Table 5.**

Table 5: Population Characteristics and Immunosuppression Therapy

Characteristics	**Results**
Age (years), mean (SD)	55 (13)
Male gender, n (%)	43 (67.2%)
Ethnic group, n (%)	
White	39 (60.9%)
Black	12 (18.8%)
Asian	13 (20.3%)
Body weight (kg), mean (SD)	76.4 (15.2)
Height (cm), mean (SD)	170.4 (8.6)
Diabetes mellitus, n (%)	16 (25%)
Time since transplantation (years)	
Mean (SD)/ Median (range)	4.1 (4.6) / 1.8 (0.3-22.8)
Donor type, n (%)	
Living / Deceased	26 (40.6%) / 38 (59.3%)
Immunosuppression at baseline:	
Tacrolimus, n (%)	
Prograf®/ Adoport®	48 (75%) / 16 (25%)
Corticosteroids, n (%)	39 (61%)
Azathioprine, n (%)	14 (21.9%)
Mycophenolate, n (%)	19 (29.7%)

4.3.1.2 Efficacy and Safety

Overall, once-daily tacrolimus was well tolerated in this study. Serum creatinine concentrations remained stable after conversion to Advagraf®. No graft losses or episodes of rejection occurred during the PK treatment period. Only six patients (9.4%) experienced a total of nine adverse events during treatment with once-daily tacrolimus. The adverse effects were vomiting (n=1), nausea (n=1), diarrhoea (n=1), headache (n=1), cough (n=2), fatigue (n=1), tongue pain (n=1), fever (n=1) and dysuria (n=1). No unexpected adverse events were reported or observed during the study period, and most adverse events were mild and transient. None of the patients discontinued due to any adverse events during the study treatments. None of the concomitant medication changed during this study.

4.3.1.3 Tacrolimus Pharmacokinetics

Before the analysis, tacrolimus pharmacokinetic parameters between Prograf® and Adoport® were compared and no significant difference was observed. Tacrolimus pharmacokinetic parameters, $AUC_{0-24,}$ C_{max}, t_{max}, C_1, C_2 and C_0 are summarised in **Table 6**. The ratios of the geometric means of C_{max}, and AUC_{0-24} were 1.01and 1.06, respectively, with 90% confidence intervals of 0.82 to 1.24 and 0.91 to 1.23 falling within the FDA acceptance interval of 0.8 – 1.25 (**Table 7**). Therefore, the two formulations were bioequivalent. However, the 90 % CI for AUC_{0-24} was outside the 90 % to 111% the EMA bioequivalence limits. This may be because we did not have a sufficient number of patients to give us sufficient power to compare the two formulations based on 90 % CI within 90-111% bioequivalence limits. However, an alternative possibility is that the two formulations are not actually bioequivalent. This is recognized as a weakness in the study. The mean time– concentration profiles for Prograf® and Adoport® are shown in **Figure 19 A**. The t_{max} of both profiles appear at different times however this may be an artefact of showing the mean of patients' profiles in each formulation. The median t_{max} was the same in both formulations.

Table 6: Prograf® and Adoport® Tacrolimus Pharmacokinetic Parameters.

Parameter	Prograf® (n=48)	Adoport® (n=16)	P-value
C_{max} (µg/L)	16.6 ± 7.7	16.6 ± 7.5	1.0
AUC_{0-24} (µg∗h/L)	191.2 ± 63.2	198.3 ± 56.3	0.5
C_0 (µg/L)	6.1 ± 1.9	6.3 ± 2.1	0.7
C_1 (µg/L)	13.6 ± 8.0	12.5 ± 8.6	0.2
C_2 (µg/L)	13.3 ± 6.7	14.7 ± 4.9	0.8
t_{max}*	2 (1-16)	2 (1-16)	0.6

Values were compared using ANOVA, general linear model (variables with a normal distribution) or the Kruskal–Wallis test (variables non-normally distributed).
*median

Table 7: Ratios of Geometric Means and 90% CI for $AUC_{0–24}$, C_{max}, for Prograf® and Adoport®

Parameter	Ratio of geometric means (%)	90% CI
$AUC_{0–24}$	106%	(91% – 123%)
C_{max}	101%	(82% – 124%)

90% CI for geometric mean are based on the ANOVA model (General linear model).

Switching from Prograf® or Adoport® to Advagraf® was made on a 1mg: 1mg basis. One hundred and twenty-eight 24-h tacrolimus PK profiles were obtained from 64 patients. The TD-Tac and OD-Tac pharmacokinetic parameters, AUC_{0-24}, C_{max}, t_{max} and C_0 are summarised in **Table 8**. Based on the mean values of non-dose-normalized data obtained on days 1 and 14, the mean tacrolimus C_{max} and C_0 were lower for Advagraf® compared to twice-daily tacrolimus. There was a significant reduction in tacrolimus C_{max} ($p = 0.028$). The mean tacrolimus C_{max} concentration fell from 16.6 µg/L SD 7.6 to 14.8 µg/L SD 5.6 with a 10% reduction [90%CI 3 to 16%]. The difference in tacrolimus C_0 was highly significant ($p = 0.009$) with a 10% reduction [90%CI 4 to 16%]. However, no significant differences were observed for AUC_{0-24} between the two formulations in this population ($p > 0.05$, Table 10). The mean concentration-time profiles for tacrolimus preparations are shown in **Figure 19 B.** The individual time–concentration profiles of the 64 patients are presented in **Figure 20**. The median t_{max} was the same for both formulations. At 90% confidence intervals (CI), the mean of C_{max} and AUC_{0-24} ratio between the twice-daily tacrolimus and Advagraf® were restricted

within the bioequivalence margin (80 –125%). The mean AUC_{0-24} Advagraf®/AUC_{0-24} twice-daily tacrolimus ratio was 96% (90% CI 91% –102%), and mean C_{max} Advagraf®/ C_{max} twice-daily tacrolimus ratio was 90% (90% CI 84% –97%). These data are summarized in **Table 11**.

Tacrolimus daily doses (mg/kg) vary considerably between individuals. To achieve a proper association between the pharmacokinetic parameters and tacrolimus formulations, these parameters were normalized to unit dose (e.g., equivalent to 0.1 mg/ kg). Dose-normalized pharmacokinetic parameters, AUC_{0-24}, C_{max}, t_{max} and C_0 are summarised in **Table 9**. There was no marked difference in dose-normalized AUC_{0-24} ($p > 0.05$). Although, dose-normalized C_{max} and dose-normalized C_0 were significantly higher for twice-daily tacrolimus compared to Advagraf® ($p < 0.05$, **Table 10**). At 90% confidence intervals (CI), dose-normalized AUC_{0-24} and dose-normalized C_{max} ratios between the twice-daily tacrolimus and Advagraf® were contained within the bioequivalence limit (80 –125%). Dose-normalized AUC_{0-24} Advagraf®/AUC_{0-24} twice-daily tacrolimus was 98% (90% CI 93% –103%) and dose-normalized C_{max} Advagraf®/ C_{max} twice-daily tacrolimus was 91% (90% CI 85% –98%, **Table 11**).

Switching from immediate to prolonged release tacrolimus had an impact on between-patient variability of tacrolimus exposure in this cohort of patients. Although tacrolimus exposure (AUC_{0-24}) tended to have less between patient variability, it was not statistically significant. The between-patient coefficients of variation (CV%) of dose-normalized C_{max}, AUC_{0-24} and C_0 for twice-daily tacrolimus (TD-Tac) were 56.8%, 66.8% and 78. 5%, respectively. Whereas, the between-patient coefficient of variation (CV%) of C_{max}, AUC_{0-24} and C_0 for Advagraf® (OD-Tac) were 48.1%, 57.3% and 65.0%, respectively (**Table 9**). **Figure 21** and **Figure 22** displayed the plot of Test for Equal Variances for dose-normalized AUC_{0-24} (µg*h/L/ mg/kg) and dose-normalized C_{max} (µg/L/mg/kg) obtained from OD-Tac and TD-Tac formulations. The plots clearly showed wider confidence interval for TD-Tac compared to OD-Tac for the $C_{max,}$ and AUC meaning that the variability is higher in the immediate release tacrolimus formulations results compared to the prolonged release tacrolimus formulation ($p=0.022$ and $p=0.085$, respectively).

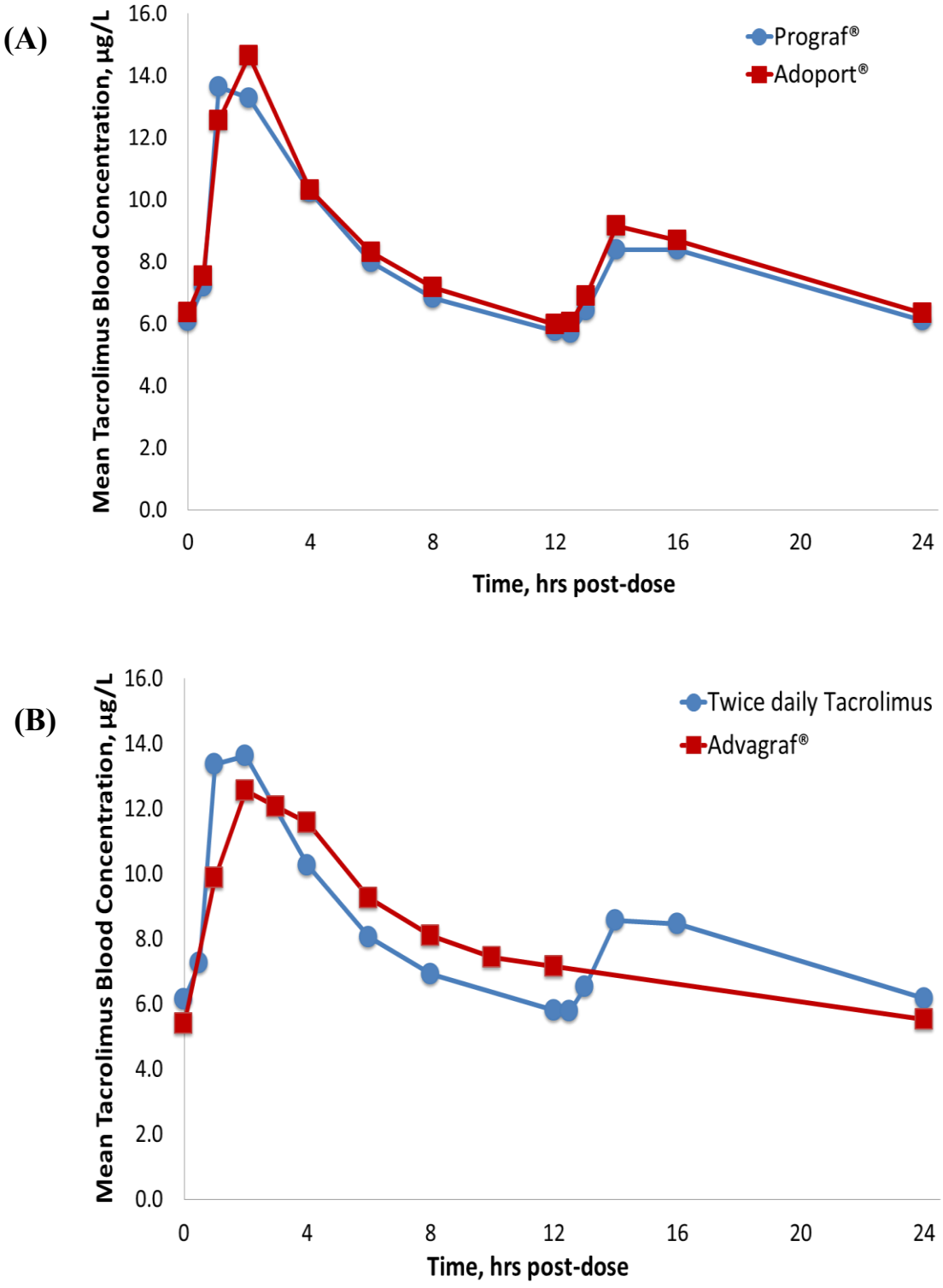
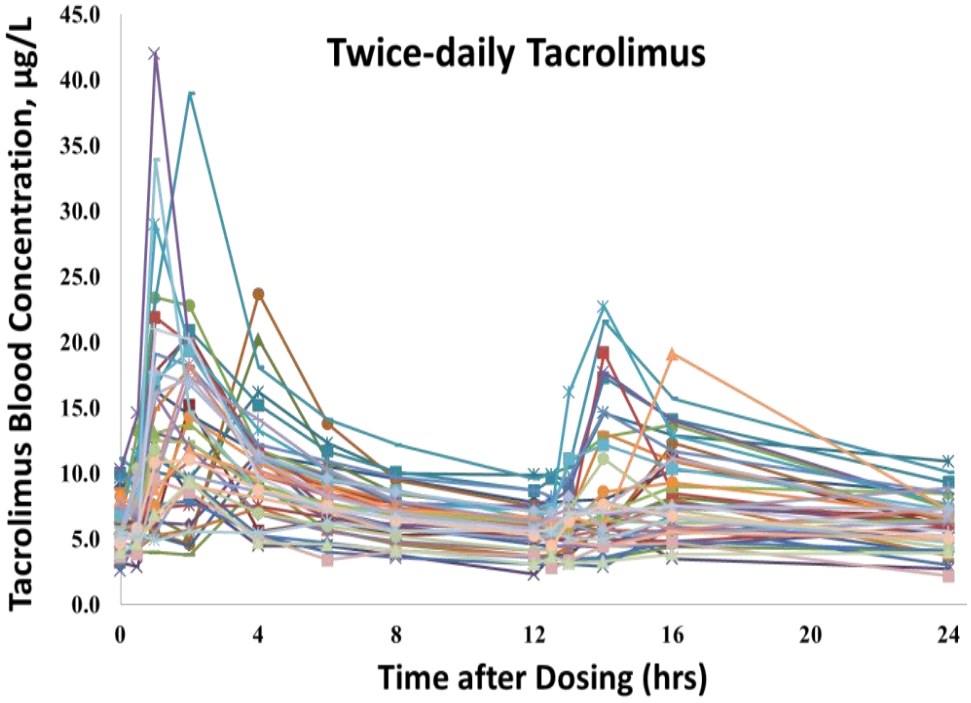


Figure 19: Time – Mean Concentration Profiles for Tacrolimus Formulations. (A) for Prograf® and Adoport® and (B) for Twice-Daily Tacrolimus and Advagraf®. Advagraf® is Equivalent to Twice-Daily Tacrolimus after a 1mg: 1mg Basis Conversion.

(A)



(B)

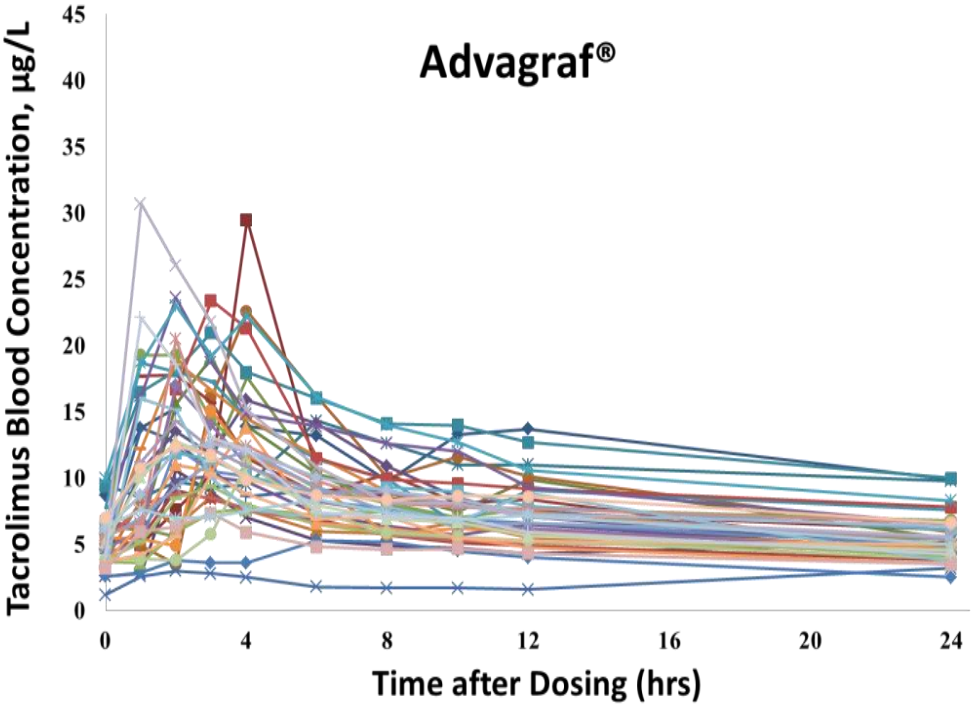


Figure 20: Whole-Blood Tacrolimus Concentration-Time Profiles in 64 Stable Kidney Transplant Recipients on Twice-Daily Tacrolimus (A) and Advagraf® (B).

Table 8: Tacrolimus Pharmacokinetic Parameters for Tacrolimus Preparations.

Patient	C_{max} (µg/L)		AUC_{0-24} (µg∗h/L)		*t_{max} (h)		C_0 (µg/L)	
	TD-Tac	OD-Tac	TD-Tac	OD-Tac	TD-Tac	OD-Tac	TD-Tac	OD-Tac
1	16.3	15.3	239	282	1	2	8.40	9.90
2	15.2	29.5	180	212	2	4	5.90	6.10
3	20.2	19.1	203	220	4	3	6.20	5.40
4	12.1	10.6	101	125	1	2	2.70	5.20
5	16.2	14.3	281	258	4	6	10.90	9.80
6	23.7	22.6	237	243	4	4	6.20	5.90
7	11.3	13.8	140	188	4	1	3.80	5.00
8	20.6	17.8	211	199	2	2	6.80	4.30
9	9.2	17.6	111	177	4	4	4.10	5.40
10	11.6	15.9	144	207	4	4	5.80	4.70
11	20.9	21.0	300	324	2	3	9.30	10.00
12	8.0	8.6	133	135	16	3	3.70	3.80
13	11.2	3.2	113	54	1	24	3.00	3.20
14	7.6	9.1	140	129	2	2	5.90	3.80
15	23.4	19.3	274	218	1	1	7.30	6.80
16	13.1	10.1	176	154	1	3	6.10	4.50
17	39.0	18.7	371	245	2	1	10.10	7.60
18	18.1	18.8	231	211	2	2	6.50	5.00
19	7.9	5.3	112	90	2	6	4.10	2.50
20	21.9	23.4	232	255	1	3	6.00	7.80
21	13.8	7.7	201	138	2	4	8.50	4.10
22	42.0	23.6	290	265	1	2	7.50	6.50
23	29.0	23.0	290	309	1	2	7.60	8.30
24	14.3	15.1	202	159	2	3	6.80	4.80
25	19.1	12.3	270	167	1	2	8.50	4.90
26	10.9	8.8	168	138	2	2	6.30	4.60
27	12.5	9.4	174	137	1	3	6.40	4.60
28	16.0	17.0	145	170	1	2	5.00	4.40
29	19.3	11.9	241	199	2	2	7.20	6.10
30	19.1	13.8	246	142	16	4	7.20	3.50
31	9.8	12.0	150	175	2	4	6.40	5.60
32	18.3	20.5	187	206	2	2	7.30	6.60
33	11.8	11.1	158	130	1	4	4.30	3.90
34	17.3	14.3	216	191	2	2	8.90	5.40
35	33.9	16.0	189	182	1	1	4.74	3.69
36	9.1	10.3	115	141	2	3	4.99	4.99
37	17.7	12.6	213	199	1	3	7.29	6.36
38	9.6	7.4	104	110	1	3	2.20	3.50
39	9.4	12.8	105	162	2	2	3.96	4.57
40	17.3	30.7	219	241	2	1	6.69	5.57
41	6.6	8.4	137	162	16	6	5.24	5.03

42	11.1	12.4	165	205	2	2	5.11	6.62
43	21.0	22.1	195	184	1	1	5.56	4.25
44	19.3	19.3	318	272	14	2	10.60	8.70
45	20.6	15.4	251	154	1	2	6.50	4.20
46	11.0	10.9	167	137	2	2	5.80	4.30
47	15.1	15.8	204	208	4	2	7.30	6.16
48	11.3	10.5	177	147	2	3	7.56	3.90
49	8.2	7.2	103	126	1	2	3.10	3.87
50	15.7	11.6	147	154	1	4	5.30	3.50
51	23.8	23.6	242	279	1	2	6.72	8.00
52	39.5	16.0	321	239	1	1	9.68	6.00
53	18.9	12.1	195	157	2	3	5.70	4.20
54	16.3	11.0	194	177	2	1	5.40	5.70
55	16.3	12.3	171	90	1	1	4.70	4.80
56	25.4	15.2	246	234	2	1	8.30	6.80
57	6.2	10.8	118	143	2	2	3.90	4.30
58	15.7	13.1	144	227	1	2	4.90	11.70
59	13.1	16.2	163	165	2	1	4.30	4.80
60	15.7	8.2	219	132	0.5	3	9.20	4.10
61	13.5	15.4	134	169	1	2	4.80	5.00
62	9.5	13.9	149	196	16	2	4.20	5.70
63	16.2	21.4	198	235	2	2	5.40	6.80
64	16.6	19.1	192	205	2	2	4.90	5.70
Mean	16.6	14.8	193.0	185.6	2.0	2.0	6.2	5.5
SD	7.6	5.6	61.2	54.4	3.8	2.9	1.9	1.8
CV%	45.7	37.6	31.7	29.3	-	-	31.4	32.8

*median

Table 9: Tacrolimus Dose-Normalized Pharmacokinetic Parameters for Tacrolimus Preparations.

Patient	C_{max} (µg/L/mg/kg)		AUC_{0-24} (µg*h/L/ mg/kg)		*t_{max} (h)		C_0 (µg/L/mg/kg)	
	TD-Tac	OD-Tac	TD-Tac	OD-Tac	TD-Tac	OD-Tac	TD-Tac	OD-Tac
1	39.7	37.2	581	687	1	2	20.4	24.1
2	30.0	58.3	356	419	2	4	11.7	12.0
3	15.8	15.0	159	172	4	3	4.9	4.2
4	9.3	8.1	77	96	1	2	2.1	4.0
5	63.3	55.8	1097	1008	4	6	42.6	38.3
6	11.5	11.0	115	118	4	4	3.0	2.9
7	12.0	14.6	148	199	4	1	4.0	5.3
8	29.1	25.2	298	281	2	2	9.6	6.1
9	6.9	11.9	83	120	4	4	3.1	3.7
10	10.5	14.4	130	187	4	4	5.2	4.3
11	27.3	27.4	391	423	2	3	12.1	13.1
12	8.8	9.4	145	148	16	3	4.1	4.2
13	21.0	6.0	211	101	1	24	5.6	6.0
14	25.5	30.5	469	433	2	2	19.8	12.7
15	18.6	15.3	218	173	1	1	5.8	5.4
16	21.1	16.3	283	249	1	3	9.8	7.3
17	45.1	26.0	429	339	2	1	11.7	10.5
18	9.1	9.4	115	106	2	2	3.3	2.5
19	62.6	42.0	883	713	2	6	32.5	19.8
20	17.5	18.7	185	204	1	3	4.8	6.2
21	73.4	41.0	1067	732	2	4	45.2	21.8
22	28.1	15.8	194	177	1	2	5.0	4.3
23	38.5	30.6	385	410	1	2	10.1	11.0
24	33.1	34.9	466	368	2	3	15.7	11.1
25	58.2	37.5	822	508	1	2	25.9	14.9
26	47.1	38.1	725	597	2	2	27.2	19.9
27	69.2	52.0	963	756	1	3	35.4	25.5
28	43.5	46.2	394	462	1	2	13.6	12.0
29	24.3	18.7	303	313	2	2	9.1	9.6
30	23.6	17.1	304	175	16	4	8.9	4.3
31	19.2	23.5	294	342	2	4	12.5	11.0
32	25.8	28.9	263	291	2	2	10.3	9.3
33	25.6	24.1	342	283	1	4	9.3	8.5
34	46.2	38.2	577	509	2	2	23.8	14.4
35	36.0	17.0	200	193	1	1	5.0	3.9
36	16.6	18.9	210	258	2	3	9.1	9.1
37	47.4	33.7	571	533	1	3	19.5	17.0
38	26.6	20.5	289	305	1	3	6.1	9.8

39	14.1	19.2	158	244	2	2	6.0	6.9
40	9.7	17.2	123	135	2	1	3.8	3.1
41	20.5	26.2	428	505	16	6	16.4	15.7
42	20.5	23.0	306	380	2	2	9.5	12.3
43	21.7	22.8	201	190	1	1	5.7	4.4
44	41.2	41.2	679	582	14	2	22.6	18.6
45	34.5	25.8	420	258	1	2	10.9	7.0
46	26.2	25.9	397	327	2	2	13.8	10.2
47	16.9	17.7	228	233	4	2	8.2	6.9
48	37.7	35.0	590	488	2	3	25.2	13.0
49	36.7	32.6	463	565	1	2	14.0	17.4
50	13.5	10.0	126	132	1	4	4.6	3.0
51	14.5	14.4	148	170	1	2	4.1	4.9
52	22.0	8.9	179	133	1	1	5.4	3.3
53	67.6	43.3	696	563	2	3	20.4	15.0
54	26.6	17.9	316	288	2	1	8.8	9.3
55	31.9	24.1	334	175	1	1	9.2	9.4
56	28.3	16.9	274	261	2	1	9.2	7.6
57	19.7	34.4	374	455	2	2	12.4	13.7
58	21.7	24.1	199	418	1	2	6.8	21.5
59	7.6	9.4	95	96	2	1	2.5	2.8
60	40.6	25.4	564	409	0.5	3	23.8	12.7
61	27.0	30.8	268	338	1	2	9.6	10.0
62	20.9	30.6	328	431	16	2	9.2	12.5
63	15.8	20.9	193	229	2	2	5.3	6.6
64	13.8	15.8	159	170	2	2	4.1	4.7
Mean	28.4	25.0	359.2	336.9	2.0	2.0	12.2	10.4
SD	16.1	12.1	240.1	192.9	3.8	2.9	9.6	6.7
CV%	56.8	48.1	66.8	57.3	-	-	78.5	65.0

*median

Table 10: Pharmacokinetic Parameters Comparison between Twice-Daily Tacrolimus (TD-Tac) and Advagraf® (OD-Tac).

Parameter	TD-Tac	OD-Tac	P-value
	Prograf® or Adoport®	Advagraf®	
C_{max} (µg/L)	16.6 ± 7.6	14.8 ± 5.6	0.028
AUC_{0-24} (µg∗h/L)	193.0 ± 61.2	185.6 ± 54.3	0.278
C_0 (µg/L)	6.2 ± 1.9	5.5 ± 1.8	0.009
Dose-normalized C_{max} (µg/L/mg/kg)	28.4 ± 16.1	25.0 ± 12.1	0.044
Dose-normalized AUC_{0-24} (µg ∗h/L/ mg/kg)	359.2 ± 240.1	336.9 ± 192.9	0.475
Dose-normalized C_0 (µg/L/mg/kg)	12.2 ± 9.6	10.4 ± 6.7	0.024

Values were compared using ANOVA (General linear model).

Table 11: Ratios of Geometric Means and 90% CI for $AUC_{0–24}$, C_{max}, Dose-Normalized $AUC_{0–24}$ and Dose-Normalized C_{max} for Advagraf® to Twice-Daily Tacrolimus

Parameter	Ratio of geometric means (%)	90% CI
$AUC_{0–24}$	96%	(91% – 102%)
C_{max}	90%	(84% – 97%)
Dose-normalized AUC_{0-24}	98%	(93% – 103%)
Dose-normalized C_{max}	91%	(85% – 98%)

90% CI for geometric mean are based on the ANOVA model (General linear model).

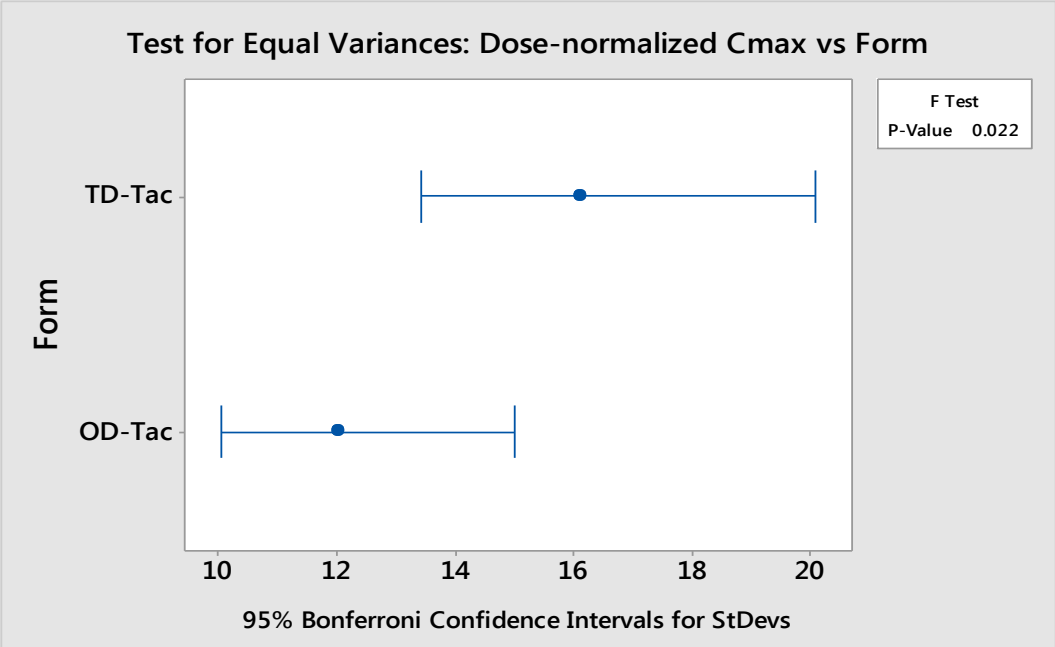


Figure 21: Plot of Test for Equal Variance with 95% Confidence Interval for C_{max} of Once- and Twice-Daily Tacrolimus. The C_{max} standard deviations for the two formulations are significantly different.

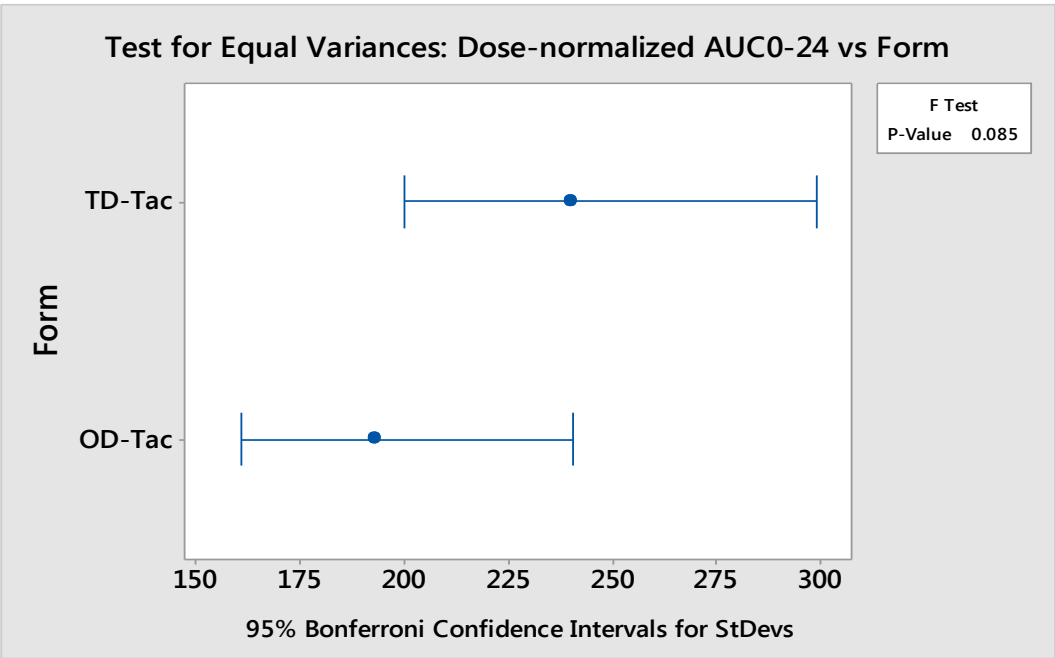


Figure 22: Plot of Test for Equal Variance with 95% Confidence Interval for AUC_{0-24} of Once-(OD-Tac) and Twice-Daily (TD-Tac) Tacrolimus. The AUC_{0-24} standard deviations for the two formulations are not significantly different.

In this study, we found a strong correlation between AUC_{0-24} and trough blood concentrations (C_0) for immediate and modified release tacrolimus at steady state, with similar correlation coefficients for both formulations (r = 0.86 and r = 0.82, respectively). The slopes of the lines of best fit were virtually identical (**Figure 23**).

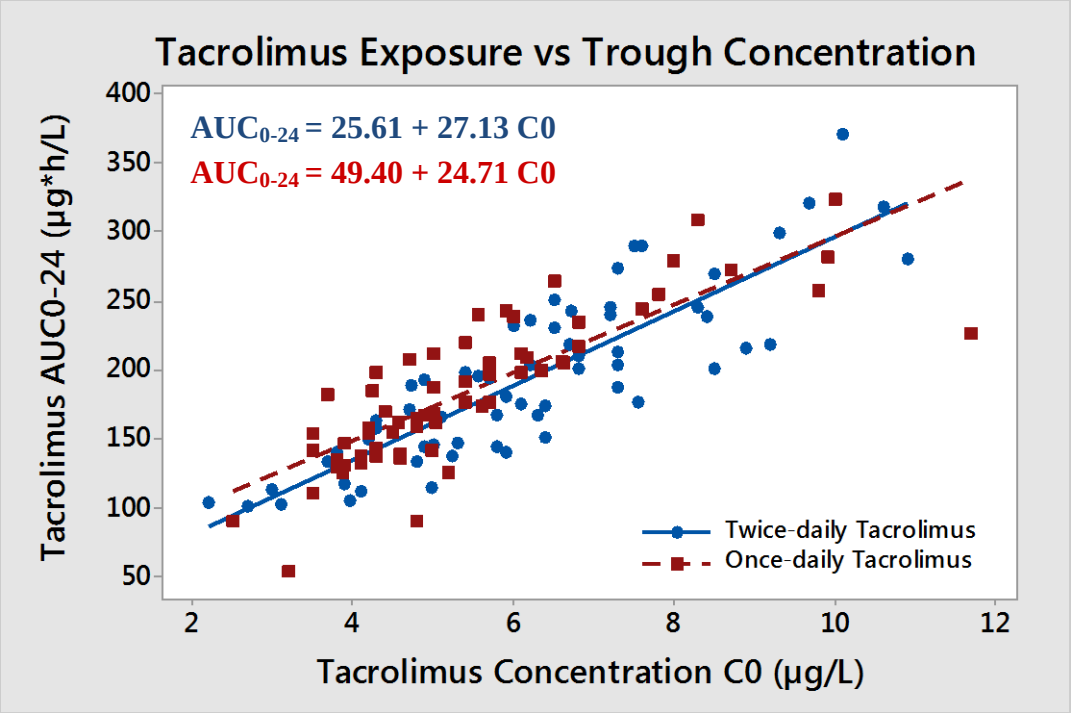


Figure 23: Scatter Plot of Individual Calculated Tacrolimus Exposure (AUC_{0-24}) vs Tacrolimus Trough Concentration (C_0) Represents the Correlation of Tacrolimus Exposure and Trough Concentration for Twice and Once-Daily Tacrolimus.

During analysis of the study samples, all the batches met the acceptance criteria for this study. Samples from these batches were successfully analysed. The quality control sample data for tacrolimus obtained during the analysis are shown in **Table 12**. The calibration curve data for tacrolimus achieved during the analysis are shown in **Table 13.**

Table 12: Quality Control (QC) Samples Achieved During Tacrolimus Analysis.

Quality Control Levels	**QC1**	**QC2**	**QC3**
Nominal Concentrations (µg/L)	**3**	**15**	**30**
Batch ID	**Measured Concentration (µg/L)**		
Batch1	2.9	14.9	29.2
Batch2	3.0	14.7	29.5
Batch3	3.0	14.5	29.9
Batch4	3.0	15.6	31.5
Batch5	2.9	14.7	31.4
Batch6	3.0	14.2	29.5
Batch7	2.9	15.5	32.2
Batch8	3.0	14.0	29.2
Batch9	2.9	14.8	29.2
Batch10	2.9	14.9	30.9
Batch11	3.0	15.0	30.6
Batch12	3.0	15.2	31.9
Batch13	3.0	15.3	31.1
Batch14	3.0	15.4	30.5
Batch15	2.9	15.3	31.3
Batch16	3.1	15.3	31.0
Batch17	2.9	14.8	30.4
Batch18	2.8	14.7	30.6
Batch19	2.8	15.0	29.4
Batch20	3.1	15.7	29.8
Batch21	3.0	16.1	32.5
Batch22	3.1	15.5	30.8
Batch23	3.1	15.7	31.0
Batch24	2.9	15.1	30.6
Batch25	2.8	14.1	27.2
Batch26	3.0	15.8	31.9
Mean	**3.0**	**15.1**	**30.5**
Standard Dev.	0.1	0.7	1.5
%CV	4.9	4.7	4.8
% Inaccuracy	-1.7	0.3	1.7
N=	111 of 111	121of 122	117 of 117

Table 13: Calibration Curve Parameters Data Achieved During Tacrolimus Analysis.

Batch ID	Curve parameters (y = A*x + B)		
	Slope (A)	**Intercept (B)**	**Correlation Coefficient (r)**
Batch1	0.016	-0.00169	0.9994
Batch2	0.0155	-0.000433	0.9996
Batch3	0.0156	-0.00129	0.9989
Batch4	0.0144	-0.000228	0.999
Batch5	0.0157	-0.00108	0.9996
Batch6	0.016	-0.00181	0.9991
Batch7	0.0153	-0.0004	0.9991
Batch8	0.0164	-0.000264	0.9992
Batch9	0.0156	-0.00087	0.9994
Batch10	0.016	-0.000864	0.9992
Batch11	0.0165	-0.00173	0.9993
Batch12	0.0158	-0.00166	0.9988
Batch13	0.0165	-0.00173	0.9993
Batch14	0.0157	-0.000523	0.9994
Batch15	0.0159	-0.000914	0.9996
Batch16	0.0156	-0.000979	0.999
Batch17	0.0153	-0.000397	0.9981
Batch18	0.0159	-0.00167	0.9981
Batch19	0.0161	-0.00206	0.9981
Batch20	0.0158	-0.000591	0.9989
Batch21	0.0142	-0.00146	0.9991
Batch22	0.0147	-0.00219	0.999
Batch23	0.016	-0.00149	0.999
Batch24	0.0161	-0.000717	0.9962
Batch25	0.0193	0.00361	0.9943
Batch26	0.0158	0.00271	0.9986
Mean	0.0158	-0.00080	0.9987
Range	0.0142 – 0.0193	-0.00219 – 0.00361	0.9943 – 0.9987

4.3.2 Associations of CYP3A5 and ABCB1 3435C>T Genotypes with Doses and Pharmacokinetics of Tacrolimus Formulations.

Patients were recruited from 2010 to 2014 in the transplant Clinic at St. George's Hospital (London, United Kingdom). 64 patients who had received twice-daily tacrolimus were converted to Advagraf® on a mg: mg basis. The patients' general demographics were shown previously in **Table 5 Page.122**. The list of the concomitant medications for 64 renal transplant patients included in the study is summarized in **Appendix 3**.

*4.3.2.1 CYP3A5*3 and ABCB1 3435C>T Genotype Analysis and Their Frequencies*

For CYP3A5 A6986G polymorphism, the melting point of the sensor probe was approximately 55°C and 61°C when hybridised to the *CYP3A5 *3/*3* (G/G) and **1/*1* (A/A) genotypes respectively. In the heterozygote polymorphism *CYP3A5*1/*3* (A/G), both melting peaks appeared (**Figure 24.A**). Moreover, for the *ABCB1* 3435C>T polymorphism, the melting point of the sensor probe was 64°C and 57°C when hybridised to *ABCB1 CC* and *TT* genotypes respectively. The heterozygote polymorphism *ABCB1 CT* presented both melting peaks (**Figure 24.B**). Twenty PCR products were randomly selected from the three genotypes melting profiles for DNA sequencing conformation. *CYP3A5*3* and *ABCB1* allelic variants results obtained by real time PCR were confirmed by sequencing these genes.

*CYP3A5*3* and *ABCB1 3435C>T* Genotypes and allele frequencies are presented in **Table 14**. In our study cohort, the *CYP3A5 *3/*3* genotype was observed in thirty-four patient (53.1%) and were thus classified as CYP3A5 non-expressers. The *CYP3A5*1/*1* and **1/*3* genotypes were observed in eighteen patients (28.1%) and twelve patients (18.8%), respectively and were classified as CYP3A5 expressers. The frequencies of the *ABCB1 CC*, *CT*, and *TT* genotypes were 15 (23.4%), 30 (46.9%), and 19 (29.7%), respectively. The *ABCB1* genotype frequency of the recipients was consistent with Hardy-Weinberg equilibrium ($p > 0.05$). Whereas, *CYP3A5*3* allele frequency deviated from Hardy–Weinberg equilibrium ($p < 0.05$).

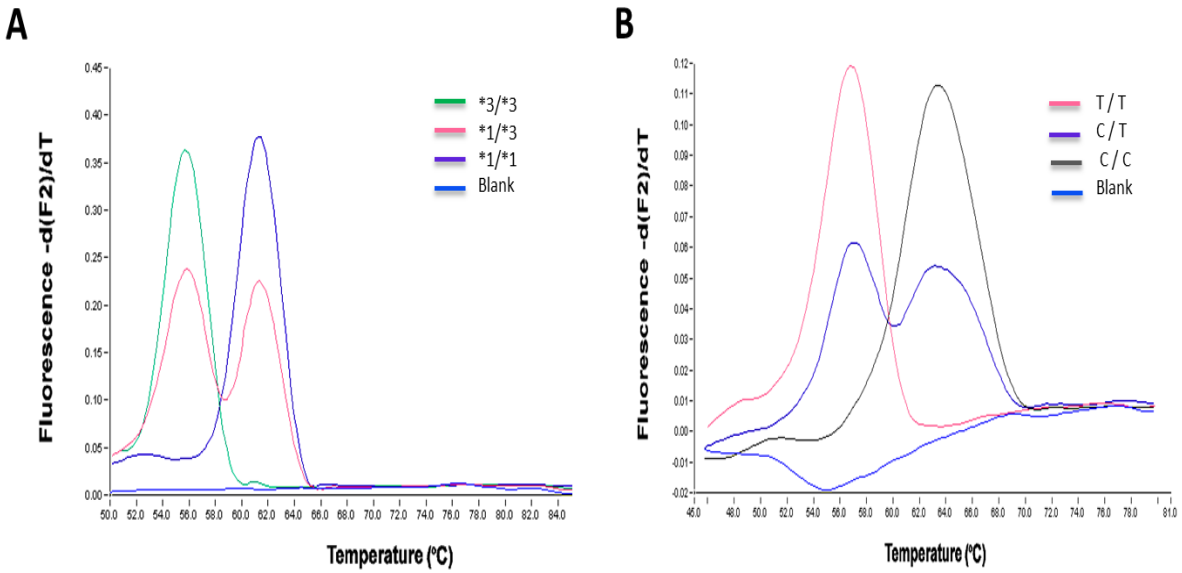


Figure 24: Derivative Melting Curve Plots for *CYP3A5*3* and *ABCB1 3435C>T* Genotyping using Specific Primers and Probes. A. The Derivative Melting Curve for the *CYP3A5* Genotypes. Samples with the Homozygous **3* Allele (; Tm 55°C); Homozygous **1* Allele (; Tm 61°C) and Heterozygous **1/*3* Genotype (; Tm 55°C and 61°C). B. The Derivative Melting Curve for the *ABCB1* Genotypes. Samples with the Homozygous *T* Allele (; Tm 57°C); Homozygous *C* Allele (; Tm 64°C) and Heterozygous *C/T* genotype (; Tm 57°C and 64°C). The Melting Curve of a no Template Control in Both Assays ( ).

Table 14: Genotype Frequencies for *CYP3A5* and *ABCB1* in Renal Transplant Patients

Gene	Variant	Genotype	n (%)	Genotype frequency (%)	Allele frequency
CYP3A5	6986A>G	**1/*1*	12	18.8%	**1* = 0.33
		**1/*3*	18	28.1%	**3* = 0.67
		**3/*3*	34	53.1%	
ABCB1	3435C>T	*C/C*	15	23.4%	C = 0.47
		C/T	30	46.9%	T = 0.53
		T/T	19	29.7%	

4.3.2.2 Association of CYP3A5 Genotypes with Tacrolimus Pharmacokinetic Profiles

Thirty patients were recruited into the CYP3A5 expressers group and 34 in the CYP3A5 non-expressers group. The baseline characteristics of the recipients are summarized in (**Table 15**). We observed no differences in these characteristics between CYP3A5 expressers and non-expressers, except for sex, diabetic status and ethnicity. In line with previous observations, the *CYP3A5*1* allele was more prevalent among black transplant recipients compared with that among white patients (**Table 15**).

Table 15: Demographic Characteristics of Patients, According to *CYP3A5* Polymorphism

Characteristic	**CYP3A5 Expressers **1/*1* (n = 12) & **1/*3*(n = 18)**	**CYP3A5 Non-expressers *3/*3* (n = 34)**
Sex		
Male/female	17/13	26/8
Age (y), mean (SD)	54.8 ± 13.3	54.4 ± 12.4
Ethnic group, n (%)		
White	10 (33.3%)	29 (85.3%)
Black	12 (40.0%)	-
Asian	8 (26.7%)	5 (14.7%)
Body weight (kg), mean (SD)	74.5 ± 12.1	78.0 ± 17.5
Height (cm), mean (SD)	170.3 ± 8.7	170.0 ± 8.5
Diabetes mellitus, n (%)	11 (36.7%)	4 (11.8%)
Time since transplantation (years)		
Mean (SD)	4.3 ± 3.8	3.9 ± 5.3
Donor type, n (%)		
Living / Deceased	7 (23.3%) / 23 (76.7%)	19 (55.9%) / 15 (44.1%)
Serum creatinine (µmol/L)	119.2 ± 39.1	127.6 ± 39.9
Serum albumin (g/L)	38.4 ± 2.7	38.8 ± 3.4
Haemoglobin (g/L)	127.7 ± 15.6	131.3 ± 27.0
Immunosuppression therapy		
Tacrolimus, n (%)		
Prograf®/ Adoport®	23 (76.7%) / 7 (23.3%)	24 (70.6%) / 10 (29.4%)
Corticosteroids, n (%)	20 (66.7%)	19 (55.9%)
Azathioprine, n (%)	7 (23.3%)	7 (20.6%)
Mycophenolate mofetil, n (%)	6 (20.0%)	13 (38.2%)

The analysis has been done on 128 24-h PK profiles obtained from 64 patients, pooling the data for TD-Tac and OD-Tac. Tacrolimus blood concentrations C_{max}, AUC_{0-24} and C_0 showed a log-normal distribution, and the data were therefore log-transformed before analysis. The *CYP3A5* SNP was a significant predictor of tacrolimus dose. Individuals possessing at least one *CYP3A5*1* allele (CYP3A5-expressers) required higher tacrolimus doses compared with *CYP3A5*3/*3* carriers (CYP3A5 non-expressers). The mean required daily dose of tacrolimus per body weight was 0.05 ± 0.03 mg/kg in the *CYP3A5*3/*3* allele carriers. This amount was significantly lower than the 0.11 ± 0.05 mg/kg (P< 0.001) of dose for *CYP3A5*1*carriers. Furthermore, we observed that the presence of the *CYP3A5*1* allele was strongly associated with lower dose-normalized tacrolimus blood concentration. CYP3A5-expressers achieved 1.8-fold lower dose-normalized tacrolimus C_{max} (19.1 ± 10.6 µg/L/mg/kg) than CYP3A5 non-expressers (33.5 ± 13.8 µg/L/mg/kg, P<0.001). In addition, *CYP3A5*1* carriers showed a significant reduction in tacrolimus dose-normalized AUC_{0-24} and C_0 than CYP3A5 non-expressers. The dose-normalized $AUC_{0–24}$ (µg*h/L per mg/kg) was significantly lower among the CYP3A5 expresser patients than *CYP3A5*3/*3* carriers (223.4 ± 140.3 vs. 458.0 ± 214.2, P<0.001). The dose-normalized C_0 was significantly lower in CYP3A5 expressers compared with that in CYP3A5 non-expressers: 6.9 ± 5.1 vs. 15.1 ± 8.7 µg/L per mg/kg, respectively (**Table 16, Figure 25**).

Table 16: Tacrolimus PK Parameters According to Their *CYP3A5*3* Genotypes for the Whole Data of Once-and Twice-Daily Tacrolimus.

PK-parameter	**CYP3A5 Expressers (**1/*1* & **1/*3*) (n= 60)**	**CYP3A5 Non-expressers (**3/*3*) (n= 64)**	**p-value**
Dose (mg/Kg/day)	0.11 ± 0.05	0.05 ± 0.03	<0.001
C_{max} (µg/L/mg/Kg)	19.1 ± 10.6	33.5 ± 13.8	<0.001
AUC_{0-24} (µg*h/L/mg/Kg)	223.4 ± 140.3	458.0 ± 214.2	<0.001
C_0 (µg/L/mg/Kg)	6.9 ± 5.1	15.1 ± 8.7	<0.001

Values were compared using ANOVA (General linear model).

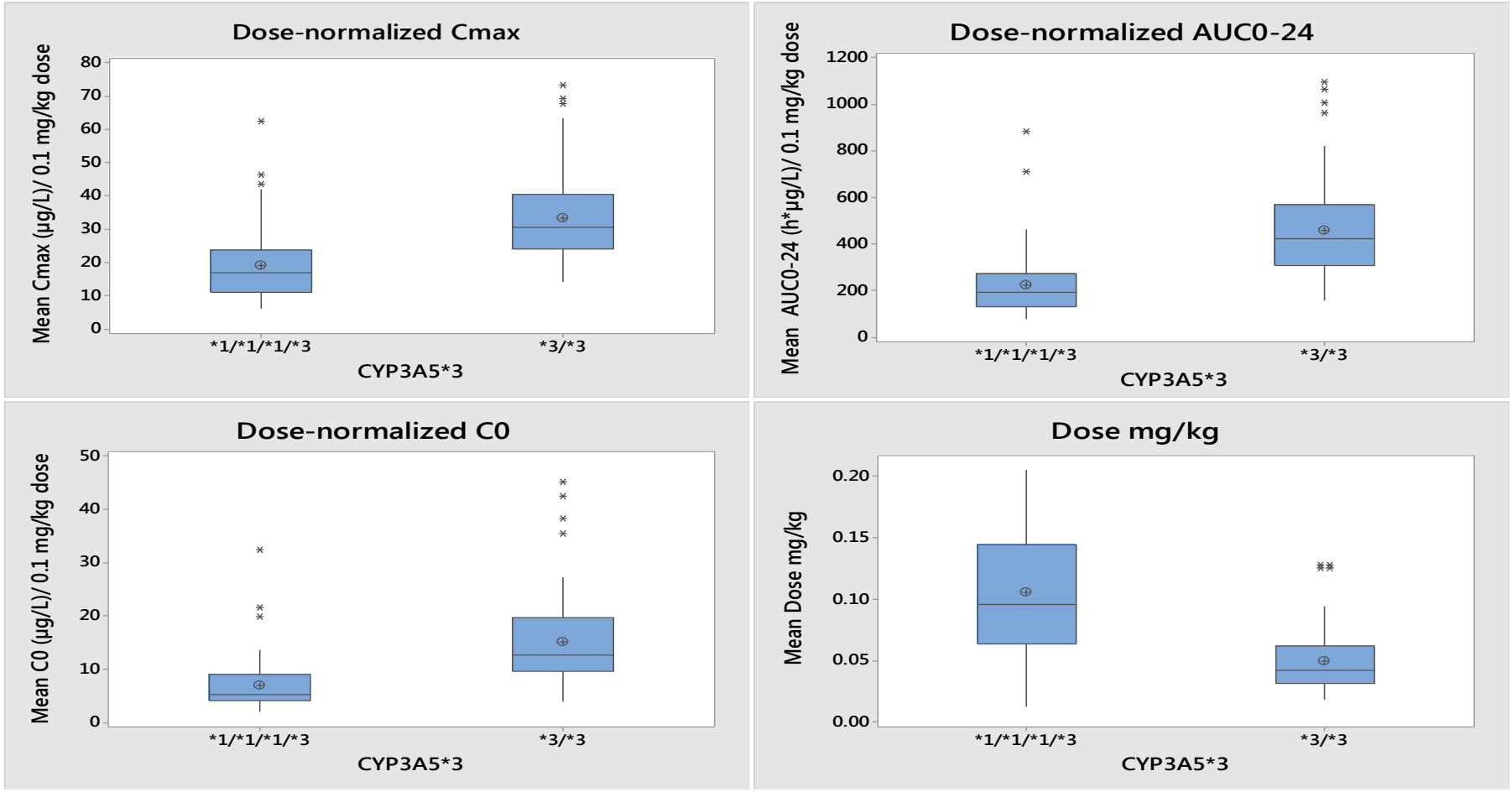


Figure 25: Associations between CYP3A5 Genotype and Tacrolimus Dose and Pharmacokinetic Parameters for the Whole Data of Once-and Twice-Daily Tacrolimus. Boxes represent the interquartile range, line in the box represents the median value, symbol in the box represents the mean value, outer lines represent the minimal and maximal value and the values beyond the whiskers (asterisks) are outliers.

Tacrolimus pharmacokinetic parameters following administration of twice-daily Prograf® or Adoport® and once-daily Advagraf® in CYP3A5 expresser and non-expresser patients are summarized in **Table 17**.

After the switch from twice to once daily tacrolimus, a slight decrease in the mean dose-normalized C_{max} and AUC_{0-24} was observed, regardless of the CYP3A5 genotype. The mean dose-normalized C_{max} and AUC_{0-24} were comparable between tacrolimus formulations in CYP3A5 expresser and non-expresser groups. In the CYP3A5 expresser group the mean dose-normalized C_0 was comparable for both formulations; however, in the CYP3A5 non-expresser patients, there was a significant reduction in the mean dose-normalized C_0 after the switch to once daily tacrolimus (twice daily tacrolimus: 16.6 ± 10.2 µg/L/mg/Kg vs Advagraf®: 13.6 ± 6.7 µg/L/mg/Kg; $P < 0.05$; **Table 17**, **Figure 26**). The blood concentration-time profiles of tacrolimus in stable kidney transplant recipients are presented in **Figure 27**.

In CYP3A5 Expressers, AUC_{0-24} OD-Tac /AUC_{0-24} TD-Tac was 101% (90% CI 93% – 110%) and C_{max} OD-Tac/ C_{max} TD-Tac was 92.5% (90% CI 81.8% –104.7%). In CYP3A5 Non-expressers, AUC_{0-24} OD-Tac /AUC_{0-24} TD-Tac was 92.5% (90% CI 86.1% –99%) and C_{max} OD-Tac/ C_{max} TD-Tac was 88.3% (90% CI 80.1% –97.4%, **Table 18**). The confidence intervals of the ratios were within the bioequivalence limits (80-125%). Therefore, the two formulations were bioequivalent. According to EMA guidelines, the confidence intervals of the AUC_{0-24} ratios were within the bioequivalence margin (90-111%) in CYP3A5 Expresser group. Consequently, the two formulations were bioequivalent only in CYP3A5 Expressers.

Table 17: Associations between *CYP3A5*3* Polymorphism and Form and Dose-Normalized Tacrolimus Pharmacokinetic Parameters.

PK-parameter	CYP3A5 Expressers (**1/*1* & **1/*3*) (n= 30)			CYP3A5 Non-expressers (**3/*3*) (n= 34)		
	TD-Tac	**Advagraf®**	**p-value**	**TD-Tac**	**Advagraf®**	**p-value**
Dose (mg/Kg/day)	0.11 ± 0.05			0.05 ± 0.03		
AUC_{0-24} (µg*h/L/mg/Kg)	223.7 ± 152.1	223.0 ± 130.1	0.72	478.7 ± 241.5	437.4 ± 184.4	0.14
C_{max} (µg/L/mg/Kg)	20.1 ± 11.8	18.1 ± 9.2	0.33	35.8 ± 15.9	31.2 ± 11	0.06
C_0 (µg/L/mg/Kg)	6.7 ± 4.5	7.2 ± 5.7	0.46	16.6 ± 10.2	13.6 ± 6.7	< 0.05

TD-Tac: Twice daily tacrolimus

Values were compared using ANOVA (General linear model).

Table 18: Ratios of Geometric Means and 90% CI for $AUC_{0–24}$ and C_{max} for Tacrolimus Formulations in *CYP3A5* Genotype Groups

Parameter	CYP3A5 Expressers (**1/*1* & **1/*3*)		CYP3A5 Non-expressers (**3/*3*)	
	Ratio of geometric means (%)	**90% CI**	**Ratio of geometric means (%)**	**90% CI**
$AUC_{0–24}$	101.3%	(93.1% – 110.1%)	92.5%	(86.1% – 99.4%)
C_{max}	92.5%	(81.8% – 104.7%)	88.3%	(80.1% – 97.4%)

90% CI for geometric mean are based on the ANOVA model (General linear model).

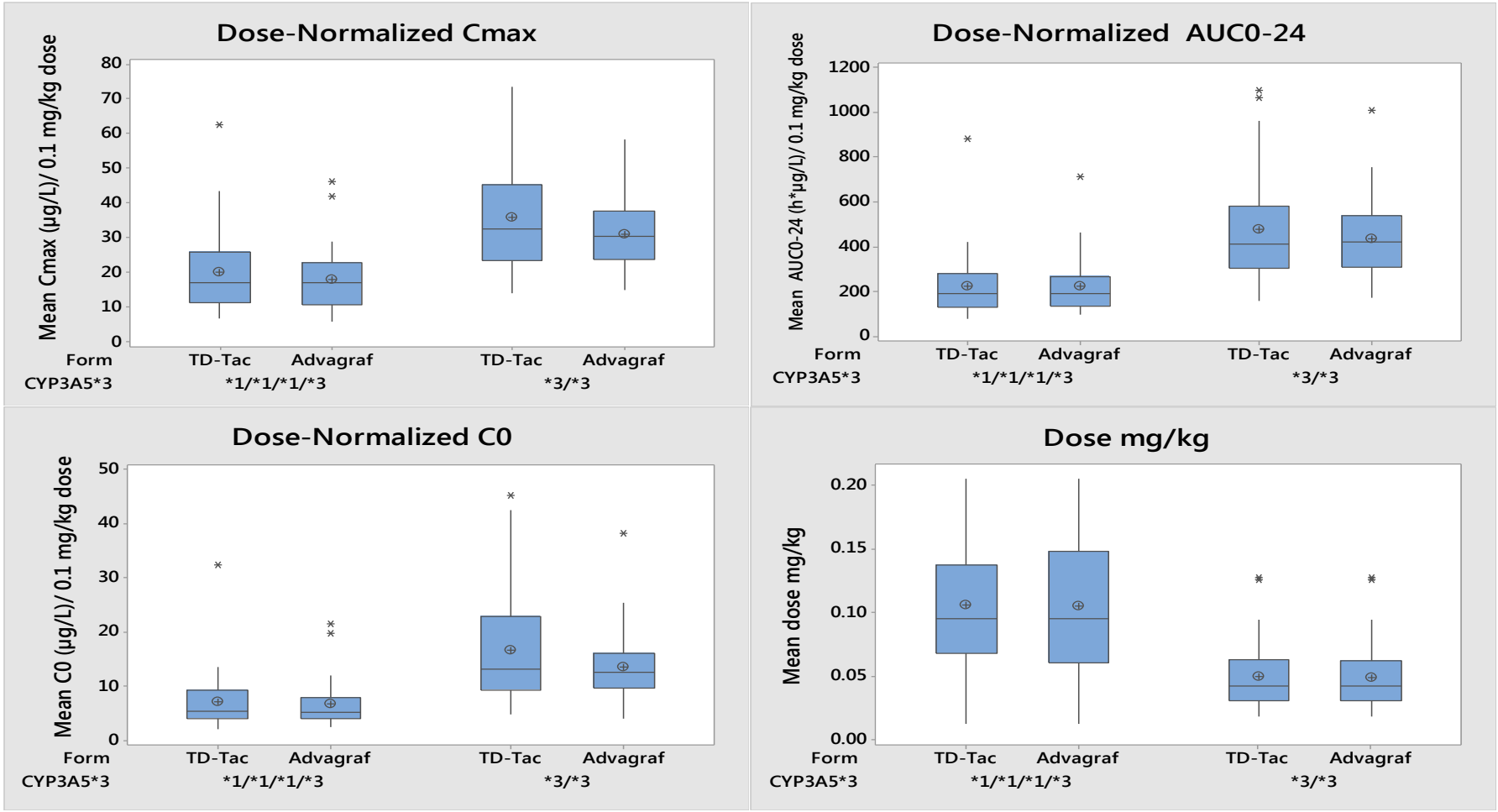


Figure 26: Associations between *CYP3A5*3* Genotype and Tacrolimus Dose-Normalized Pharmacokinetic Parameters of Twice-Daily Tacrolimus (TD-Tac) and Advagraf®. Boxes represent the interquartile range, line in the box represents the median value, symbol in the box represents the mean value, outer lines represent the minimal and maximal value and the values beyond the whiskers (asterisks) are outliers.

A

B

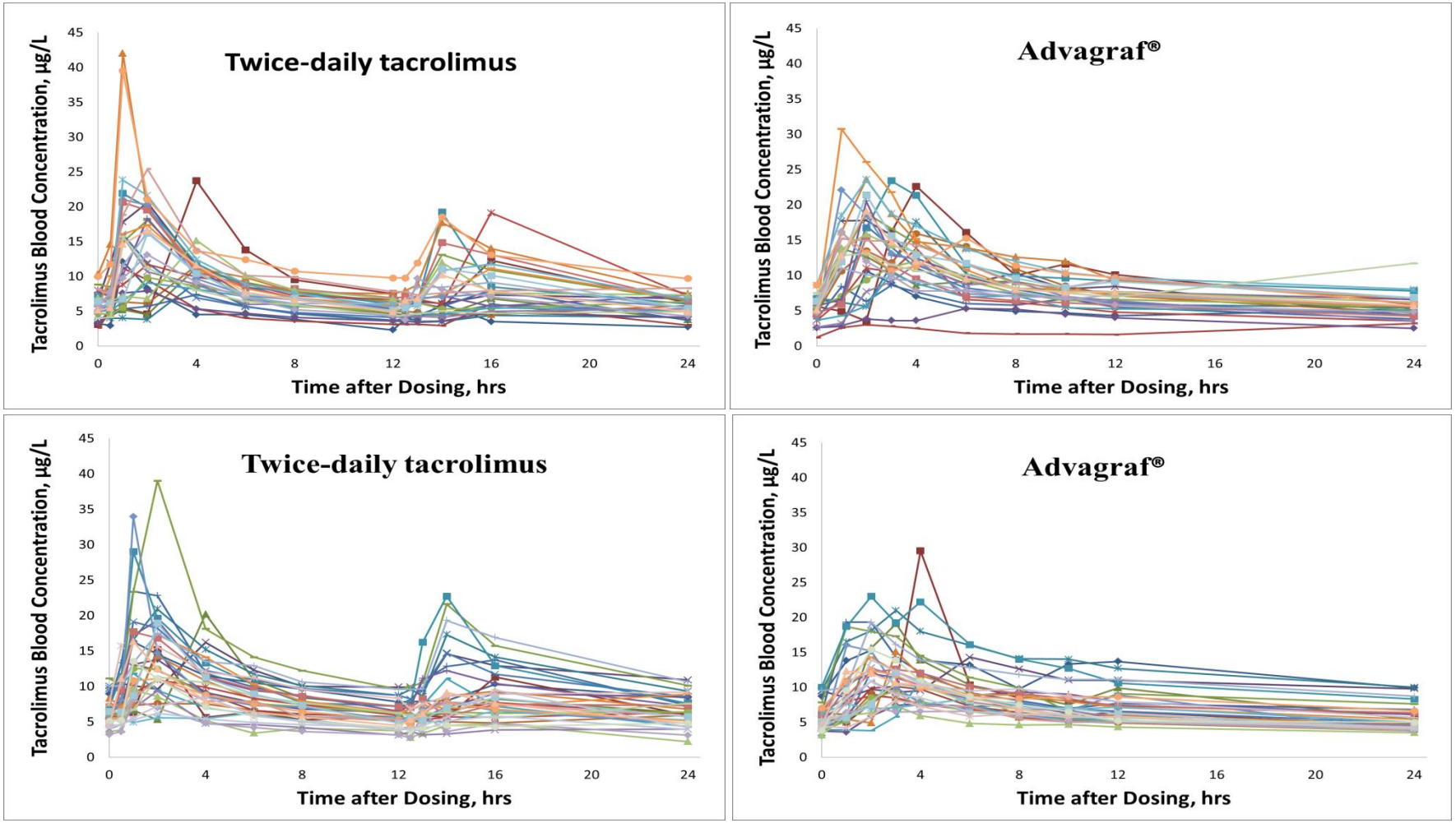


Figure 27: Summary of Blood Tacrolimus Concentration Time Profiles in Stable Adult Kidney Transplant Recipients on Twice-Daily Tacrolimus (before the Switch) and Advagraf® (after the Switch) for *CYP3A5 *1* allele carriers (A) and *CYP3A5 *3*3* carriers (B).

4.3.2.3 Associations between ABCB1 3435C>T Variants and Tacrolimus Pharmacokinetic Profiles

A total of 64 patients was included. The demographic characteristics of the *ABCB1 CC* as well as the *ABCB1 CT/TT* carriers included in our study are presented in **Table 19.** There were no significant differences between the *ABCB1 CC* carriers and *ABCB1 CT/TT* carriers, except for haemoglobin content, ethnicity and the proportion of the donor type.

Table 19: Demographic Characteristics of Patients, According to *ABCB1 3435C>T* Polymorphism

Characteristic	***ABCB1 CC* carriers (n= 16)**	***ABCB1 CT/TT* carriers CT (n= 29) & TT (n= 19)**
Sex		
Male/female	10/6	33/15
Age (y), mean (SD)	55.4 ± 13.1	54.3 ± 12.7
Ethnic group, n (%)		
White	5 (31.3%)	34 (73%)
Black	9 (56.3%)	3 (6%)
Asian	2 (12.5%)	11 (23%)
Body weight (kg), mean (SD)	77.2 ± 16.5	76.1 ± 14.9
Height (cm), mean (SD)	173.3 ± 8.8	169.0 ± 8.2
Diabetes mellitus, n (%)	3 (18.8%)	12 (25%)
Time since transplantation		
Mean (SD)	3.3 ± 2.7	4.4 ± 5.1
Donor type, n (%)		
Living / Deceased	4/12	22/26
Serum creatinine (µmol/L)	125.3 ± 48.9	124.2 ± 36.8
Serum albumin (g/L)	38.1 ± 1.9	39.1 ± 3.4
Haemoglobin (g/L)	123.7 ± 20.9	131.6 ± 22.6
Immunosuppression therapy		
Tacrolimus, n (%)		
Prograf®/ Adoport®	14 (81.3%) / 2 (12.5%)	33 (69%) / 15 (31%)
Corticosteroids, n (%)	11 (69%)	28 (58%)
Azathioprine, n (%)	1 (6%)	13 (27%)
Mycophenolate mofetil, n (%)	7 (44%)	12 (25%)

The analysis has been done on 128 24-h PK profiles obtained from 64 patients pooling the data for TD-Tac and OD-Tac. The tacrolimus pharmacokinetic parameters according to *ABCB1 3435C>T* polymorphisms are shown in **Table 20**. A significant difference was observed at dose-normalized tacrolimus pharmacokinetic parameters (C_{max}, AUC_{0-24} and C_0) between the *ABCB1 CC* and the *CT/TT* genotypes. The dose-normalized C_{max} (µg/L/mg/kg) was significantly lower among the *ABCB1* CC genotype than the *ABCB1 T* allele carriers (20.9 ± 14.2 vs. 28.7 ± 13.9, P<0.001). *ABCB1 CC* patients showed reduced dose-normalized AUC_{0-24} than *ABCB1 CT/TT* patients (248.6 ± 184.7 vs. 381.2 ± 218.0 µg*h/L/mg/kg, P<0.001). A significantly lower dose-normalized C_0 was achieved in patients homozygous for CC genotype (7.4 ± 5.9 µg/L/mg/kg) compared with patients carrying at least one T allele (12.5 ± 8.6 µg/L/mg/kg, P<0.001). Moreover, we found that recipients with CC genotype required a higher tacrolimus dose compared to those with *CT/TT* genotypes (0.10 ± 0.05 vs. 0.07 ± 0.04, P<0.001, **Table 20 & Figure 28**).

Table 20: Tacrolimus Dose-Normalized PK Parameters According to *ABCB1 3435C>T* Genotypes for the Whole Data of Once-and Twice-Daily Tacrolimus.

PK-parameter	*ABCB1 CC* (n= 32)	*ABCB1 CT/TT* (n= 96)	p-value
Dose (mg/Kg/day)	0.10 ± 0.05	0.07 ± 0.04	<0.001
C_{max} (µg/L/mg/Kg)	20.9 ± 14.2	28.7 ± 13.9	<0.001
AUC_{0-24} (µg*h/L/mg/Kg)	248.6 ± 184.7	381.2 ± 218.0	<0.001
C_0 (µg/L/mg/Kg)	7.4 ± 5.9	12.5 ± 8.6	<0.001

Values were compared using ANOVA (General linear model).

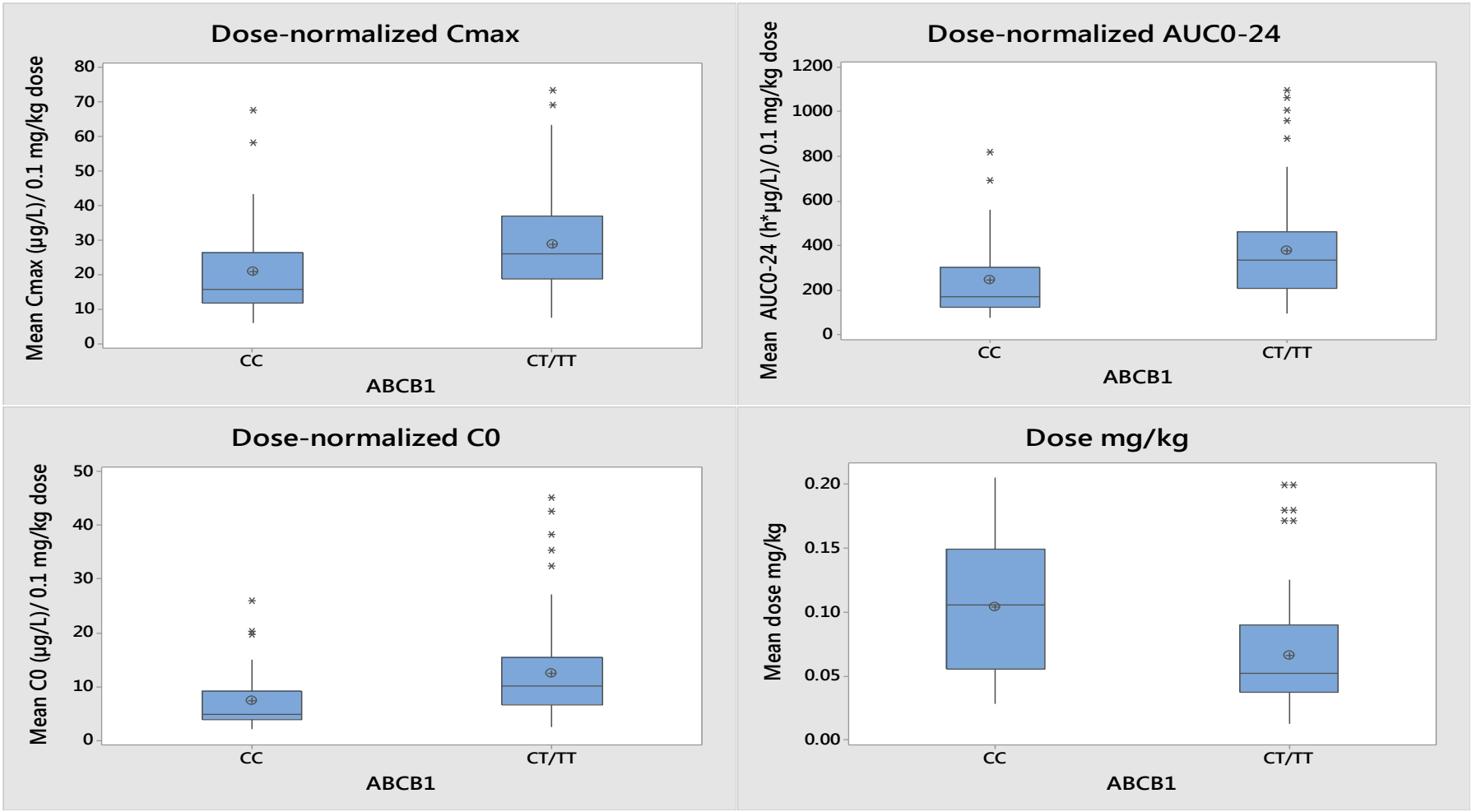


Figure 28: Associations between *ABCB1 3435C>T* Polymorphisms and Tacrolimus Dose and Dose-Normalized Pharmacokinetic Parameters for the Whole Data of Once-and Twice-Daily Tacrolimus. Boxes represent the interquartile range, line in the box represents the median value, symbol in the box represents the mean value, outer lines represent the minimal and maximal value and the values beyond the whiskers (asterisks) are outliers.

Table 21 shows tacrolimus exposure parameters following administration of twice-daily tacrolimus and once-daily Advagraf® in *ABCB1 CC* and *ABCB1 CT/TT* groups. In both *ABCB1* groups, there was a slight decrease in tacrolimus dose-normalized C_{max}, AUC_{0-24} and C_0 after the switch to once-daily tacrolimus. Regardless of the *ABCB1 3435C>T* genotype group, the mean dose-normalized C_{max}, as well as mean dose-normalized AUC_{0-24} and C_0 were comparable for both tacrolimus formulations (P >0.05; **Table 21, Figure 29**). The blood concentration-time profiles of tacrolimus in stable kidney transplant recipients are presented in **Figure 30**.

The ratio of means (90% CI) of $AUC_{0–24}$ for OD-Tac versus TD-Tac was as follows: *ABCB1 CC* carriers, 94.3% (82.1% –108.2 %); *ABCB1 CT/TT* carriers, 97.3% (91.7% – 103.2%). While the ratio of means (90% CI) of C_{max} for OD-Tac versus TD-Tac was as follows: *ABCB1 CC* carriers, 85.4% (70.0% –104.3%); *ABCB1 CT/TT* carriers, 92.0% (84.9% –99.7%). As the OD-Tac/TD-Tac C_{max} ratio in the *ABCB1 CC* group is outside the 90% CI of 80, 125, the two formulations were not bioequivalent. According to EMA guidelines, the confidence intervals of the AUC_{0-24} ratios were within the bioequivalence margin (90-111%) in *ABCB1 CT/TT* genotype group. Consequently, the two formulations were bioequivalent only in *ABCB1 CT/TT* carriers. These data are summarized in **Table 22**.

Table 21: Relationship of *ABCB1 3435C>T* Polymorphism and Tacrolimus Formulation with Dose-Normalized Tacrolimus Pharmacokinetic Parameters.

PK-parameter	*ABCB1 CC* (n= 15)			*ABCB1 CT/TT* (n= 49)		
	TD-Tac	**Advagraf®**	**P-value**	**TD-Tac**	**Advagraf®**	**P-value**
Dose (mg/Kg/day)	0.10 ± 0.06	0.10 ± 0.06		0.07 ± 0.04	0.07 ± 0.04	
C_0 (µg/L/mg/Kg)	8.3 ± 7.2	6.5 ± 4.1	0.18	13.5 ± 10.0	11.6 ± 7.0	0.07
C_{max} (µg/L/mg/Kg)	23.3 ± 17.1	18.5 ± 10.5	0.16	30.1 ± 15.6	27.2 ± 11.8	0.15
AUC_{0-24} (µg*h/L/mg/Kg)	268.0 ± 219.0	229.2 ± 147.5	0.41	389.6 ± 241.2	372.8 ± 194.1	0.79

TD-Tac: Twice daily tacrolimus.

Values were compared using ANOVA (General linear model).

Table 22: Ratios of Geometric Means and 90% CI for $AUC_{0–24}$ and C_{max} for Tacrolimus Formulations in *ABCB1 3435C>T* Genotype Groups.

Parameter	*ABCB1 CC*		*ABCB1 CT/TT*	
	Ratio of geometric means (%)	**90% CI**	**Ratio of geometric means (%)**	**90% CI**
$AUC_{0–24}$	94.3%	(82.1% – 108.2 %)	97.3%	(91.7% – 103.2%)
C_{max}	85.4%	(70.0% – 104.3%)	92.0%	(84.9% – 99.7%)

90% CI for geometric mean are based on the ANOVA model (General linear model).

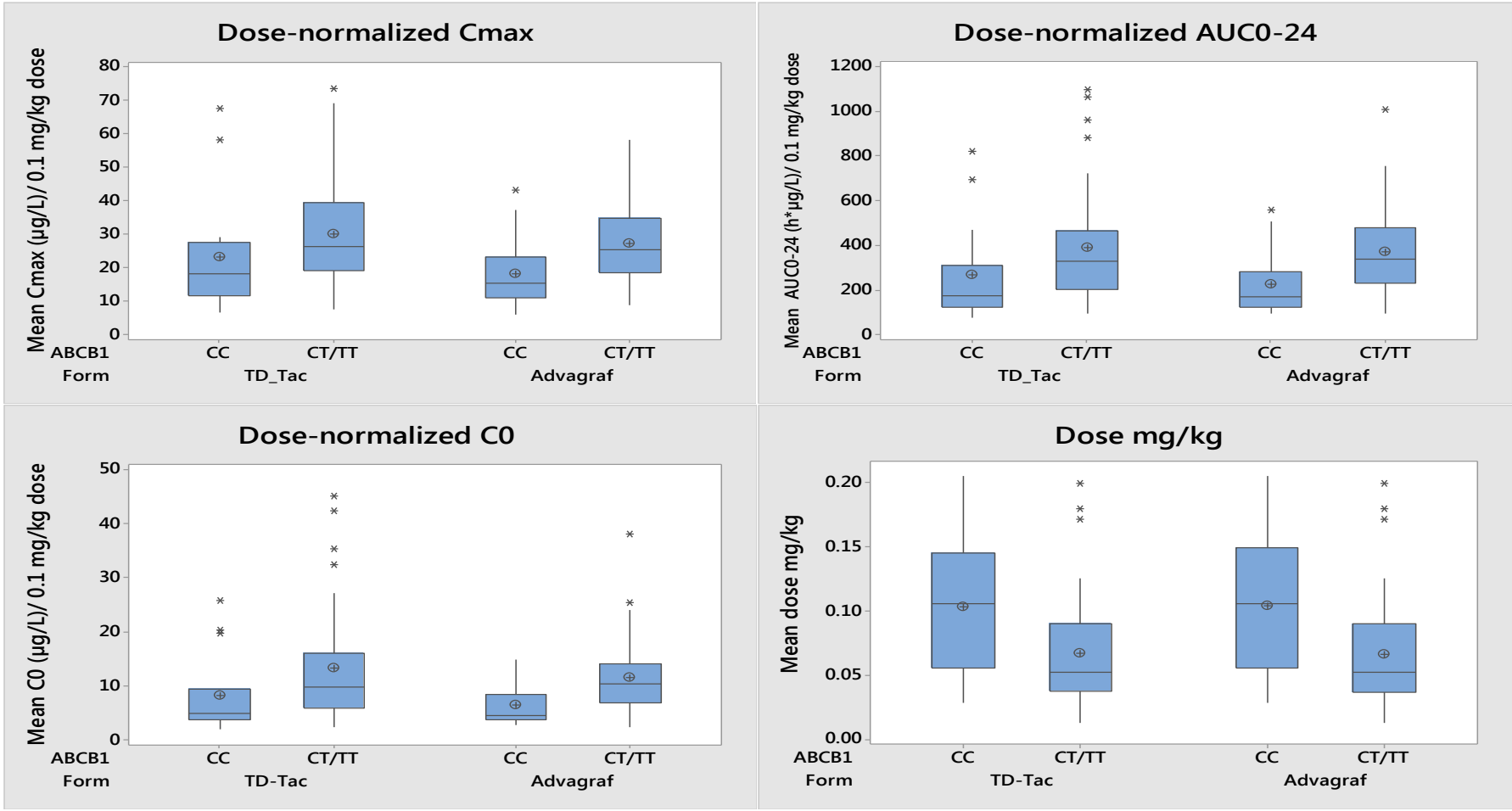


Figure 29: Associations between *ABCB1 3435C>T* Polymorphism and Tacrolimus Dose-Normalized Pharmacokinetic Parameters of Twice-Daily Tacrolimus (TD-Tac) and Advagraf®. Boxes represent the interquartile range, line in the box represents the median value, symbol in the box represents the mean value, outer lines represent the minimal and maximal value and the values beyond the whiskers (asterisks) are outliers.

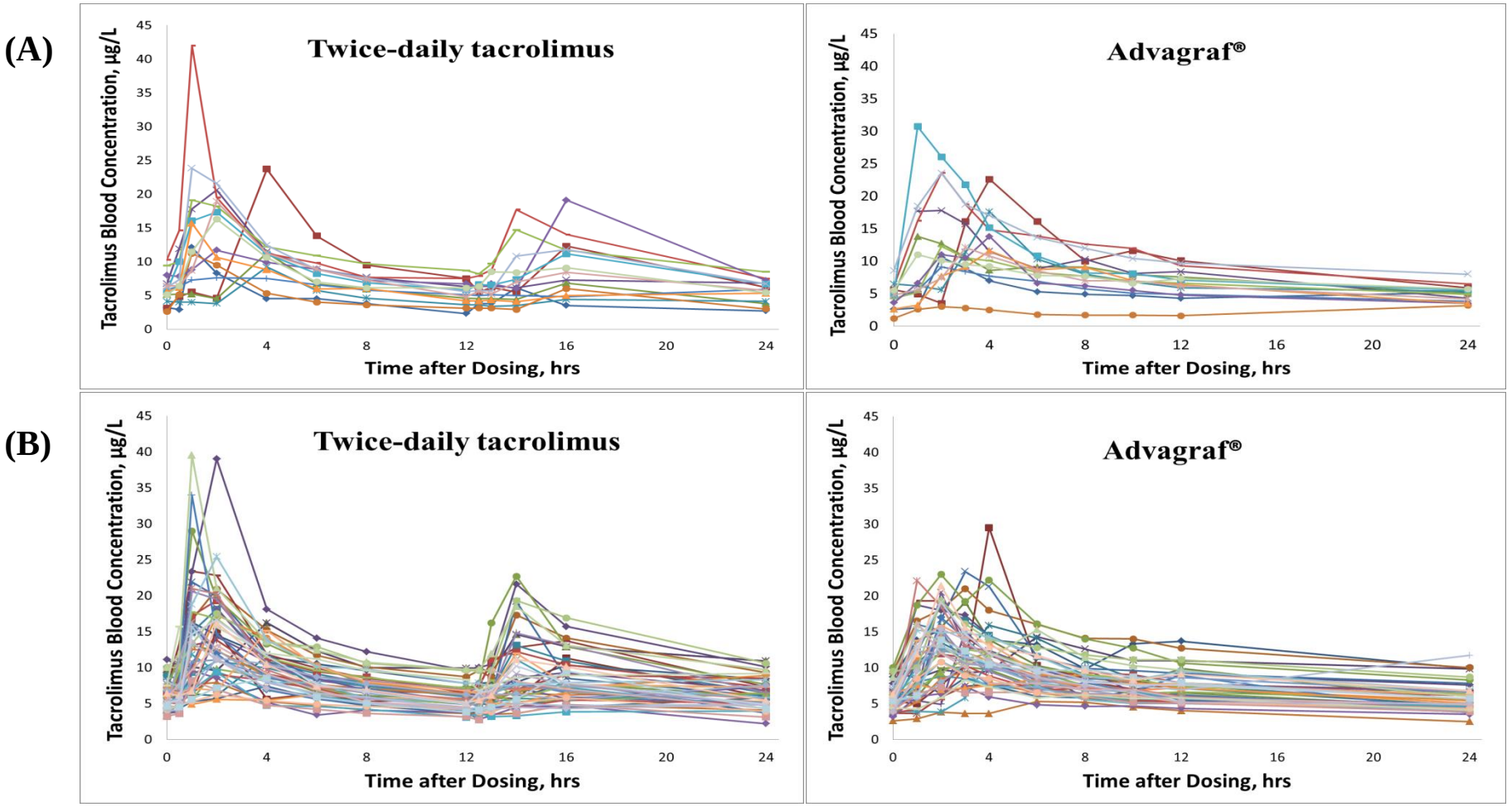


Figure 30: Summary of Blood Tacrolimus Concentration Time Profiles in Stable Adult Kidney Transplant Recipients on Twice-Daily Tacrolimus (before the Switch) and Advagraf® (after the Switch) for *ABCB1 CC* (A) and *ABCB1 CT/TT* (B) Groups.

*4.3.2.4 Associations between CYP3A5*3 and ABCB1 3435C>T Combined Genotypes and Tacrolimus Pharmacokinetic Profiles*

On the basis of the results described above, we subsequently divided all the patients, according to both *CYP3A5*3* and *ABCB1 3435C>T* genotypes. 4 genotype combinations were identified in our study population: CYP3A5 expresser recipients (*CYP3A5*1*allele carriers) with *ABCB1 CC* genotype (n=12); CYP3A5 expresser recipients with *ABCB1 CT/TT* genotype (n=18); CYP3A5 non-expresser recipients (*CYP3A5*3/*3* carriers) with *ABCB1 CC* genotype (n=4) and CYP3A5 non-expresser recipients with *ABCB1 CT/TT* genotype (n=30). *CYP3A5*3*3* and *ABCB1 CC* genotype group, with 4 patients only, was excluded from the statistical analysis. The demographic and baseline clinical characteristics of the study participants are presented in **Table 23**. Demographic characteristics, except ethnicity ($p< 0.001$), donor type ($p< 0.05$) and diabetic status ($p< 0.01$) were not significantly different between the genotype groups. The analysis has been done on 128 24-h PK profiles obtained from 64 patients with data for TD-Tac and OD-Tac pooled.

After excluding the four subjects with *CYP3A *3*3* and *ABCB1 CC* genotype from the analysis, significant differences in tacrolimus pharmacokinetic parameters were observed between groups. The difference in these parameters increased progressively from CYP3A5 expresser /*ABCB1CC* group to CYP3A5 non-expresser/*ABCB1 CT/TT* group. No significant difference was noted in CYP3A5 non-expressers between *ABCB1 CT/TT* and *ABCB1CC* carriers (the excluded group). When CYP3A5 non-expresser/*ABCB1 CT/TT* group compared with CYP3A5 expressers/*ABCB1CC* and *CT/TT* genotype subgroup, significant differences in tacrolimus dose requirements, dose-normalized $AUC_{0–24}$, dose-normalized C_{max} and dose-normalized C_0 were evident. CYP3A5 non-expresser/*ABCB1 CT/TT* group required significantly lower tacrolimus dose 0.05 mg/kg/day (SD: 0.02) compared with CYP3A5 expresser/*ABCB1 CT/TT* group who needed 0.10 (SD: 0.05) mg/kg/day ($P<0.001$) and CYP3A5 expresser/*ABCB1 CC*, who needed 0.12 (SD: 0.05) mg/kg/day ($P<0.001$). In CYP3A5 expresser/*ABCB1 CT/TT* group, tacrolimus dose was significantly lower than CYP3A5 expresser/*ABCB1 CC* group ($P=0.03$). CYP3A5 non-expresser */ABCB1 CT/TT* carriers

developed a significantly higher dose-normalized C_0 than *CYP3A5* expresser */ABCB1 CT/TT* carriers ($p<0.001$) and then *CYP3A5* expresser */ABCB1 CC* carriers ($p<0.001$). CYP3A5 expresser/*ABCB1 CC* group had a significantly lower tacrolimus dose-normalized C_0 compared with CYP3A5 expresser/*ABCB1 CT/TT* group ($P<0.01$). A significant difference was observed in tacrolimus dose-normalized C_{max} in CYP3A5 non-expresser/*ABCB1 CT/TT* group compared with CYP3A5 expresser/*ABCB1 CT/TT* ($p<0.001$) and CYP3A5 expresser/*ABCB1 CC* groups ($p<0.001$). Tacrolimus dose increase significantly in CYP3A5 expresser/*ABCB1 CT/TT* group compared with CYP3A5 expresser/*ABCB1 CC* group ($P=0.02$). In addition, CYP3A5 non- expresser */ABCB1 CT/TT* carriers achieved 2.6-fold higher dose-normalized AUC_{0-24} compared with CYP3A5 expresser/*ABCB1 CT/TT* and 1.8-fold higher dose-normalized AUC_{0-24} than CYP3A5 expresser */ABCB1 CC* carriers. A significant difference was observed in tacrolimus dose-normalized $AUC_{0–24}$ between the *ABCB1 CC* and the *CT/TT* genotype subgroup ($P<0.05$; **Figure 31; Table 24**).

Table 23: Patients Demographic Characteristics According to *CYP3A5* and *ABCB1 3435C>T* Genotypes.

Characteristic	CYP3A5 Expressers (**1/*1 & *1/*3*)		CYP3A5 Non-expressers (**3/*3*)	
	ABCB1 CC	***ABCB1 CT/TT***	***ABCB1 CC***	***ABCB1 CT/TT***
Sex				
Male/female	7/5	10/8	3/1	23/7
Age (y) , mean (SD)	55.8 ± 15.0	54.1 ± 12.4	54.3 ± 5.1	54.4 ± 13.1
Ethnic group, n (%)				
White	2 (17%)	8 (44%)	3 (75%)	26 (87%)
Black	9 (75%)	3 (17%)	0 (0%)	0 (0 %)
Asian	1 (8%)	7 (39%)	1 (25%)	4 (13 %)
Body weight (kg), mean (SD)	78.0 ± 16.4	72.2 ± 8.0	74.7 ± 19.3	78.5 ± 17.5
Height (cm), mean (SD)	174.2 ± 9.2	167.7 ± 7.5	170.6 ± 7.6	169.9 ± 8.7
Diabetes mellitus, n (%)	2 (17%)	9 (50%)	1 (25%)	3 (10%)
Time since transplantation (years)				
Mean (SD)	3.7 ± 2.7	4.7 ± 4.4	1.9 ± 2.5	4.2 ± 5.6
Donor type, n (%)				
Living / Deceased	1/11	6/12	3/1	16/14
Serum creatinine (µmol/L)	120.9 ± 51.5	119.6 ± 31.1	138.3 ± 43.7	126.8 ± 40.0
Serum albumin (g/L)	38.4 ± 1.9	38.8 ± 3.1	37.0 ± 1.4	39.3 ± 3.7
Haemoglobin, g/L	124.0 ± 20.4	130.2 ± 11.4	122.8 ± 25.4	132.4 ± 27.4
Immunosuppression therapy				
Tacrolimus, n (%)				
Prograf®/ Adoport®	11 (92%) /1 (8%)	12 (67%) / 6 (33%)	3 (75%) / 1 (25%)	21 (70%) / 9 (30%)
Corticosteroids, n (%)	10 (83%)	11 (61%)	2 (50%)	17 (57%)
Azathioprine, n (%)	1 (8%)	6 (33%)	0 (0%)	7 (23%)
Mycophenolate mofetil, n (%)	5 (42%)	1 (6%)	2 (50%)	11 (37%)

Table 24: Tacrolimus Dose-Normalized PK Parameters According to *CYP3A5*3* and *ABCB1 3435C>T* Genotypes for the Whole Data of Once-and Twice-Daily Tacrolimus.

PK-parameter	CYP3A5 Expressers (**1/*1 & *1/*3*)		CYP3A5 Non-expressers (**3/*3*)		p-value [a]	p-value [b]	p-value [c]
	ABCB1 CC **(n= 12)**	***ABCB1 CT/TT*** **(n= 18)**	***ABCB1 CC*** **(n= 4)**	***ABCB1 CT/TT*** **(n= 30)**			
Dose (mg/Kg/day)	0.12 ± 0.05	0.10 ± 0.05	0.05 ± 0.05	0.05 ± 0.02	<0.001	<0.001	<0.05
C_{max} (µg/L/mg/Kg)	15.6 ± 6.8	21.4 ± 12.0	36.7 ± 19.0	33.1 ± 13.1	<0.001	<0.001	<0.05
AUC_{0-24} (µg*h/L/mg/Kg)	172.3 ± 74.4	257.4 ± 163.0	477.6 ± 230.3	455.4 ± 213.9	<0.001	<0.001	<0.05
C_0 (µg/L/mg/Kg)	5.0 ± 2.2	8.2 ± 6.0	14.7 ± 7.5	15.1 ± 8.9	<0.001	<0.001	<0.01

[a] p-Values refer to comparisons between CYP3A5 Expressers / *ABCB1 CC* group and CYP3A5 non-expresser/*ABCB1 CT/TT* group.
[b] p-Values refer to comparisons between CYP3A5 Expressers/ *ABCB1 CT/TT* group and CYP3A5 non-expresser/*ABCB1 CT/TT* group.
[c] p-Values refer to comparisons between CYP3A5 Expressers subgroups.

Values were compared using ANOVA (General linear model).

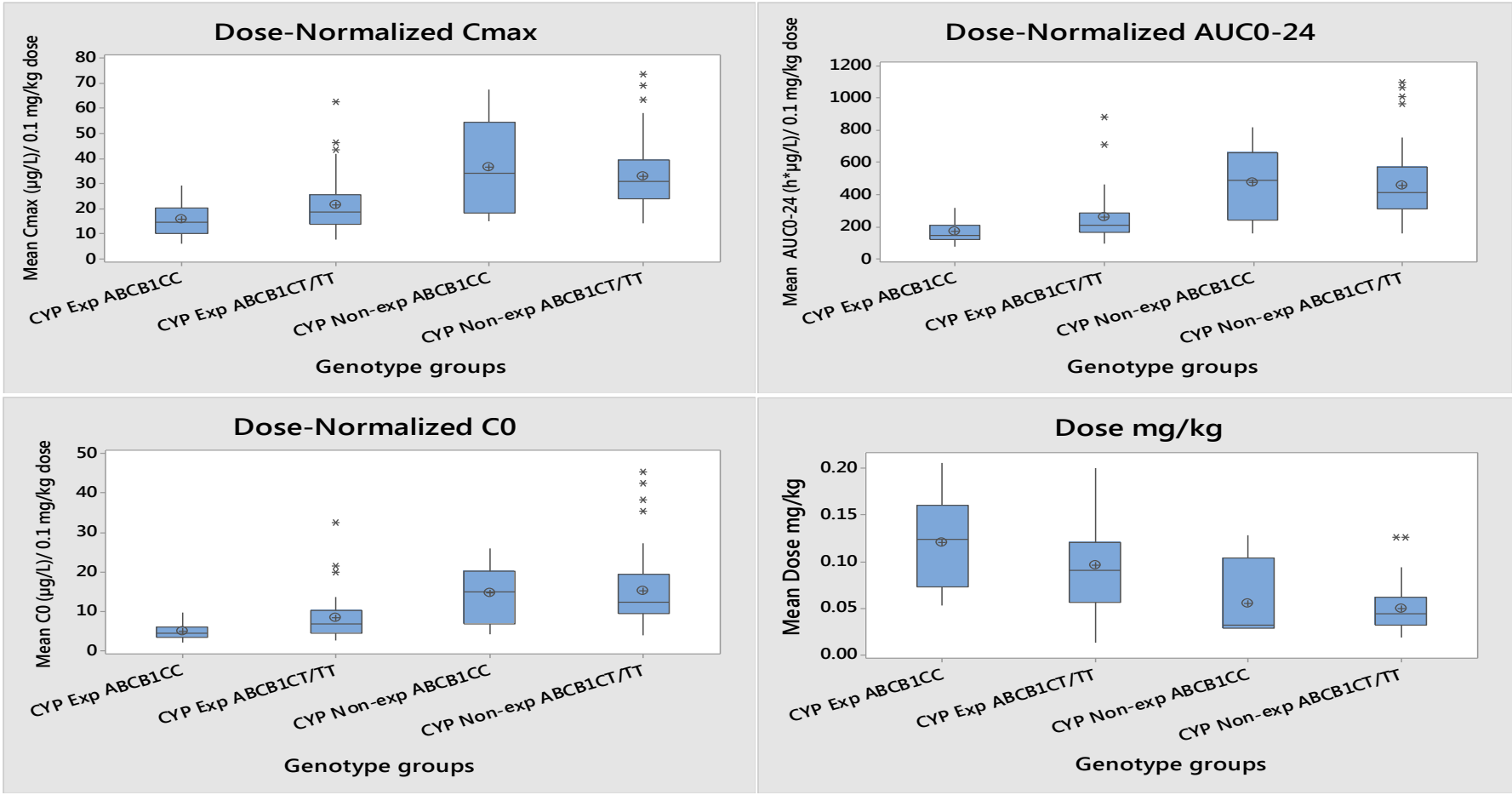


Figure 31: *CYP3A5* and *ABCB1 3435C>T* Combined Genotypes and Dose-Normalized Tacrolimus Exposure and Dose Requirement for the Whole Data of Once-and Twice-Daily Tacrolimus. Boxes represent the interquartile range, line in the box represents the median value, symbol in the box represents the mean value, outer lines represent the minimal and maximal value and the values beyond the whiskers (asterisks) are outliers. CYP Exp refers to CYP3A5 expressers (*CYP3A5*1/*1* and **1/*3*) and CYP Non-exp refers to CYP3A5 non-expressers (*CYP3A5*3/*3*).

Additionally, when we compared changes in these different genotype groups by formulation, no difference was found between twice-daily tacrolimus and Advagraf® in mean weight-adjusted dose, dose-normalized C_0, dose-normalized C_{max} and dose-normalized $AUC_{0\text{-}24}$ between the different genotype groups (**Figure 32** & **Table 25**). The blood concentration-time profiles of tacrolimus are presented in **Figure 33.**

In CYP3A5 Expressers *ABCB1 CC* group, the ratio of OD-Tac/TD-Tac for $AUC_{0–24}$ was 97.9% with a 90% CI of 82.3% – 116.6% and for C_{max} was 86.3% with a 90% CI of 66.4% – 112.3%. In CYP3A5 Expressers *ABCB1 CT/TT* group, the ratio of the OD-Tac/TD-Tac for $AUC_{0–24}$ was 103.5% with a 90% CI of 94.5% – 113.4% and for C_{max} was 96.6% with a 90% CI of 85.2% – 110.2%. While in the CYP3A5 Non-expressers *ABCB1 CT/T* group, the ratio of OD-Tac/TD-Tac was as follows for $AUC_{0–24}$ was 86.7% (90% CI of 93.7% – 101.3%) and for C_{max} was 89.1% (90% CI of 80.1% – 99.1%, **Table 26**). As the OD-Tac/TD-Tac C_{max} ratio in CYP3A5 expressers *ABCB1 CC* group is outside the 90% CI of 80, 125, both formulations were not bioequivalent. Whereas the other groups OD-Tac/TD-Tac ratios for $AUC_{0–24}$ and C_{max} are within the 90% CI of 80, 125, bioequivalence was achieved with both tacrolimus formulations. According to EMA guidelines, the confidence intervals of the $AUC_{0\text{-}24}$ ratios were outside the bioequivalence margin (90-111%) except in CYP3A5 expressers *ABCB1 CT/TT* carriers. Therefore both formulations were bioequivalent only in CYP3A5 Expresser *ABCB1CT/TT* carriers.

Table 25: Tacrolimus Dose-Normalized Pharmacokinetic Parameters for Different Combination of *CYP3A5*3* and *ABCB1 3435C>T* Genotypes in Both Once- and Twice-Daily Tacrolimus.

PK-parameter	CYP3A5 Expressers (**1/*1* & **1/*3*)					
	ABCB1 CC (n= 12)			*ABCB1 CT/TT* (n= 18)		
	TD-Tac	**Advagraf®**	**P-value**	**TD-Tac**	**Advagraf®**	**P-value**
Dose (mg/Kg/day)	0.12 ± 0.05	0.12 ± 0.05		0.10 ± 0.05	0.10 ± 0.05	
C_0 (µg/L/mg/Kg)	5.3 ± 2.6	4.7 ± 1.8	0. 7	8.5 ± 6.8	8.0 ± 5.4	0. 6
C_{max} (µg/L/mg/Kg)	17.2 ±8.0	14.1 ± 5.2	0.3	22.0 ± 13.7	20.8 ±10.4	0.8
AUC_{0-24} (µg*h/L/mg/Kg)	178.0 ± 86.0	166.0 ± 64.0	0.8	253.8±179.6	261.0 ± 9.7	0.4
PK-parameter	**CYP3A5 Non-expressers (**3/*3*)**					
	ABCB1 CC (n= 4)			*ABCB1 CT/TT* (n= 30)		
	TD-Tac	**Advagraf®**	**P-value**	**TD-Tac**	**Advagraf®**	**P-value**
Dose (mg/Kg/day)	0.05 ± 0.05	0.05 ± 0.05		0.05 ± 0.02	0.05 ± 0.02	
C_0 (µg/L/mg/Kg)	17.7 ± 9.0	11.7 ± 5.1	0.03	16.4 ± 10.5	13.9 ± 6.9	0.06
C_{max} (µg/L/mg/Kg)	41.8 ± 25.0	31.6 ± 12.2	0.3	35.0 ± 14.8	31.1±11.0	0.1
AUC_{0-24} (µg*h/L/mg/Kg)	536 ± 291	418.8 ± 172.8	0.2	471.0 ± 238.9	439.9 ± 188.5	0.3

Values were compared using ANOVA (General linear model).

Table 26: Ratios of Geometric Means and 90% CI for $AUC_{0–24}$ and C_{max} for Tacrolimus Formulations in CYP3A5 and *ABCB1 3435C>T* Genotype Groups

Parameter	CYP3A5 Expressers (**1/*1* & **1/*3*)				CYP3A5 Non-expressers (**3/*3*)	
	ABCB1 CC		*ABCB1 CT/TT*		*ABCB1 CT/TT*	
	Ratio of geometric means (%)	90% CI	Ratio of geometric means (%)	90% CI	Ratio of geometric means (%)	90% CI
$AUC_{0–24}$	97.9%	(82.3% – 116.6%)	103.5%	(94.5% – 113.4%)	93.7%	(86.7% – 101.3%)
C_{max}	86.3%	(66.4% – 112.3%)	96.6%	(85.2% – 110.2%)	89.1%	(80.1% – 99.1%)

90% CI for geometric mean are based on the ANOVA model (General linear model).

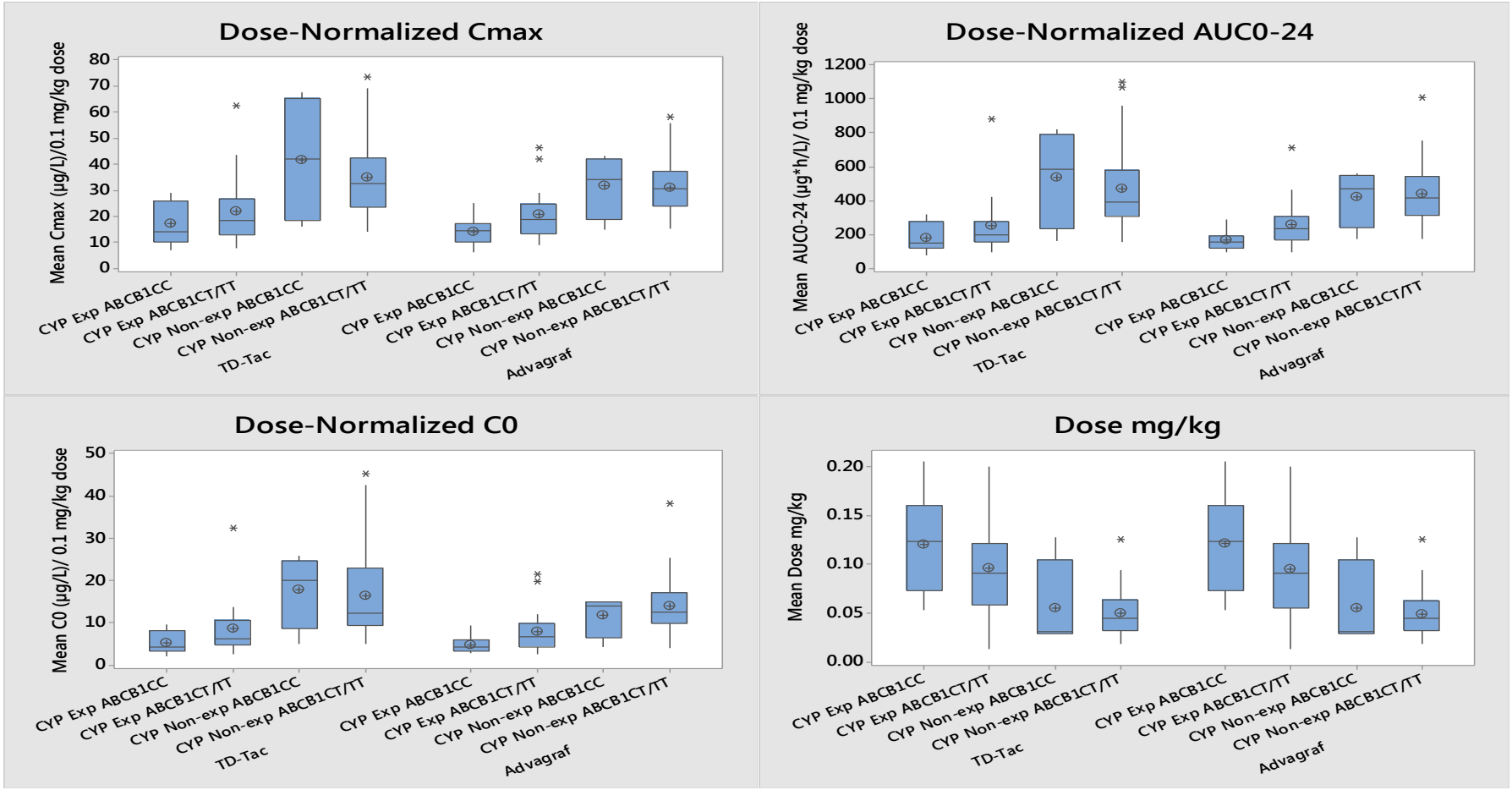


Figure 32: Associations of *CYP3A5* and *ABCB1 3435C>T* Genotypes with Tacrolimus Dose-Normalized Pharmacokinetic Parameters of Twice-Daily Tacrolimus and Advagraf®. Boxes represent the interquartile range, line in the box represents the median value, symbol in the box represents the mean value, outer lines represent the minimal and maximal value and the values beyond the whiskers (asterisks) are outliers. CYP Exp refers to CYP3A5 expressers (*CYP3A5*1/*1* and **1/*3*) and CYP Non-exp refers to CYP3A5 non-expressers (*CYP3A5*3/*3*).

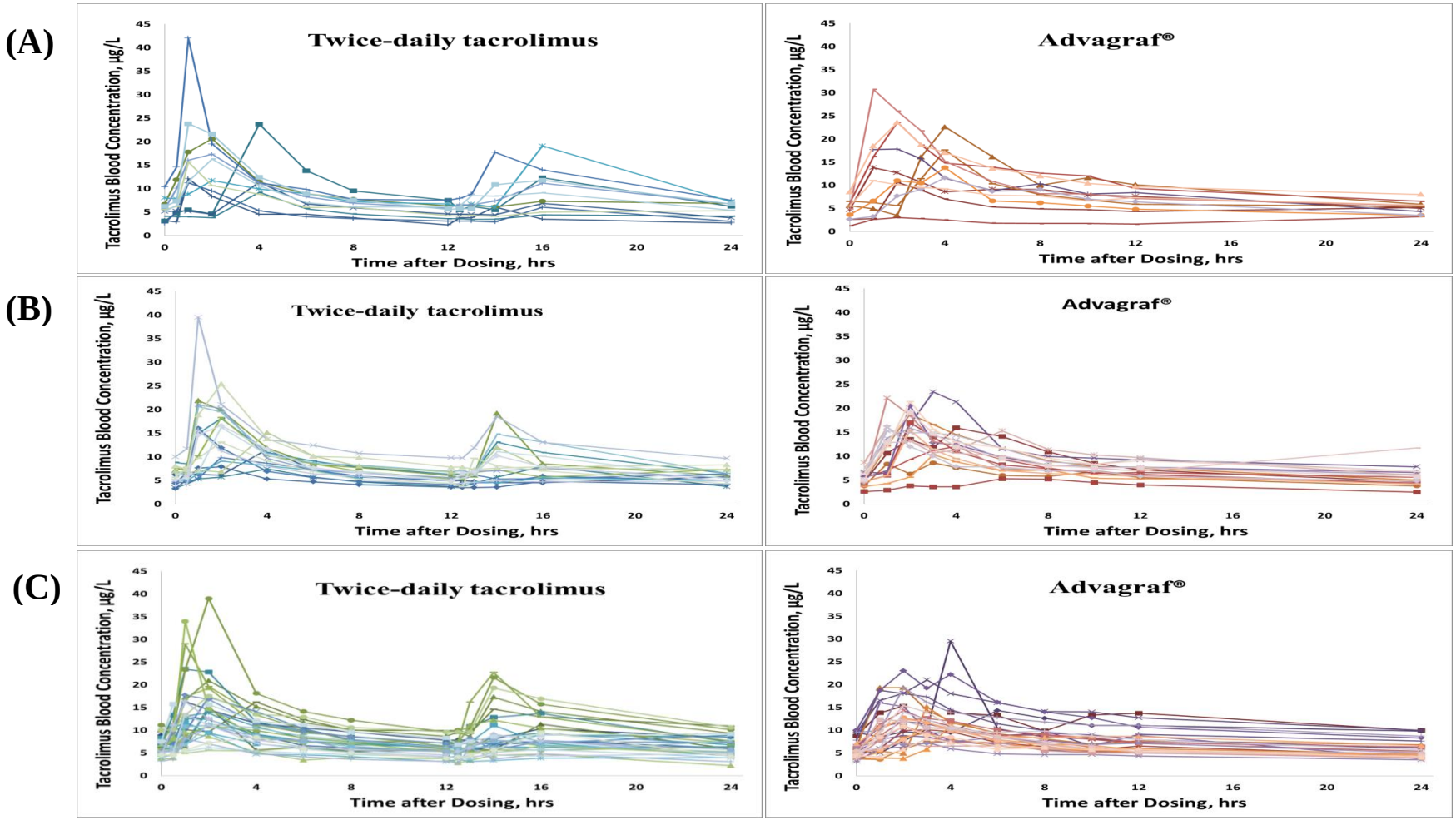


Figure 33: Summary of Blood Tacrolimus Concentration Time Profiles for CYP3A5 Expressers (*CYP3A5*1/*1* and **1/*3*) / *ABCB1 3435 CC* (A), CYP3A5 Expressers (*CYP3A5*1/*1* and **1/*3*) /*ABCB1 CT/TT* (B) and CYP3A5 non-Expressers (*CYP3A5*3/*3*) /*ABCB1 CT/TT* (C).

4.3.2.5 Factors Associated with Dose Requirements of Tacrolimus

Factors associated with dose requirements of tacrolimus were studied using univariate regression analysis. The analysis has been done on 128 24-h PK profiles obtained from 64 patients with data for TD-Tac and OD-Tac pooled. The P value was highly significant with ethnicity, sex, *CYP3A5*3* genotype and the combined *CYP3A5* and *ABCB1* genotype ($p<0.001$ for each) and haematocrit and *ABCB1* genotype ($p=0.001$ for each). The P values for age and time since transplant were less than 0.05. Although diabetic patients had higher tacrolimus dose-normalized C_{max} and required lower tacrolimus dose than non-diabetic patients, the difference was not statistically significant. The p-value was 0.098 and 0.056, respectively. The *CYP3A5*3* genotype explains 33.9% of the variability in tacrolimus dose requirements. Similarly, the combined *CYP3A5* and *ABCB1* genotype accounts for 36.9% of the between-individual variability in tacrolimus dose requirements.

Multiple regression analysis by stepwise selection identified the combined *CYP3A5*3* and *ABCB1* genotype, age, sex, ethnicity, haematocrit, corticosteroids treatment and diabetic status as independent variables associated with tacrolimus dose (**Table 27**). These factors explain 59.9% of the variability in tacrolimus dose requirements. After adjusting for these independent predictors of tacrolimus dose, *CYP3A5/ABCB1* combined genotype remained strongly associated with tacrolimus dose ($p <0.001$) and accounts for 36.9% of tacrolimus dose requirements variability. Moreover, patient diabetic status became strongly associated with tacrolimus dose ($p=0.001$) and responsible for 9.0% of the variability in tacrolimus dose requirements. Besides, patient sex effect was not statistically significant ($P >0.05$, **Table 27**).

Regarding dose-normalized C_0, multiple regression analysis by stepwise selection identified the combined *CYP3A5*3* and *ABCB1* genotype, haematocrit, tacrolimus formulation and diabetic status as independent variables associated with tacrolimus C_0 (**Table 27**). These factors explain 40.1% of the variability in tacrolimus trough concentration. The contributions of the individual variables are shown in **Table 27**.

CYP3A5/ABCB1 combined genotype had a strong relationship with tacrolimus dose ($p < 0.001$) and accounts for 26.0% of the variability in tacrolimus trough concentration.

For dose-normalized $AUC_{0-24,}$ multiple regression analysis by stepwise selection identified the combined *CYP3A5*3* and *ABCB1* genotype, sex, age, Time since transplantation, donor type and diabetic status as independent variables associated with tacrolimus AUC_{0-24} (**Table 27**). These factors explain 49.4% of the variability in tacrolimus exposure (AUC_{0-24}). The contributions of the individual variables are shown in **Table 27**. *CYP3A5/ABCB1* combined genotype had a strong association with tacrolimus dose ($p < 0.001$) and accounts for 31.1% of tacrolimus exposure (AUC_{0-24}) variability.

Table 27: Factors Associated with Tacrolimus Exposure and Dose Requirements (Multiple Regression Analysis).

Stepwise regression equation	**R^2**	**Independent variables**
Dose (mg/kg) = 0.2199 - 0.000622 * Age - 0.1636 * Haematocrit - 0.0387 (if Asian) - 0.0217 (if White) - 0.02665 (if diabetic) + 0.01043 (if treated with corticosteroids) + 0.00974 (if female) + 0.0017 (if *CYP3A5*1/*1/*1/*3 /ABCB1CT/TT* genotype) - 0.0457 (if *CYP3A5*3/*3 /ABCB1CC* genotype) - 0.0534 (if *CYP3A5*3/*3 /ABCB1CT/TT* genotype).	59.9%	Age ($p = 0.011$, $R^2 = 1.8\%$) Sex ($p = 0.137$, $R^2 = 0.8\%$) Corticosteroids ($p = 0.081$, $R^2 = 1.2\%$) Ethnicity ($p = 0.004$, $R^2 = 4.7\%$) Haematocrit ($p = 0.01$, $R^2 = 5.6\%$) Diabetic status ($p = 0.001$, $R^2 = 9.0\%$) *CYP3A5*/*ABCB1* genotype ($p < 0.001$, $R^2 = 36.9\%$)
Dose-Normalized C_0 (µg/L/ mg/kg) = -12.66 + 44.9 *Haematocrit + 4.98 (if diabetic) + 1.69 (if *CYP3A5*1/*1/*1/*3 /ABCB1CT/TT* genotype) + 10.31 (if *CYP3A5*3/*3 /ABCB1CC* genotype) + 10.07 (if *CYP3A5*3/*3 /ABCB1CT/TT* genotype) - 1.82 (if formulation _Advagraf®).	40.1%	Formulation ($p = 0.12$, $R^2 = 1.2\%$) Diabetes ($p = 0.001$, $R^2 = 5.5\%$) Haematocrit ($p < 0.001$, $R^2 = 7.3\%$) *CYP3A5*/*ABCB1* genotype ($p < 0.001$, $R^2 = 26.0\%$)
Dose-Normalized AUC_{0-24} (µg*h/L/ mg/mL) = -154.8 + 3.36*Age + 111.4*(if living donor type) + 105.6 (if diabetic) + 9.70* time since transplantation (y) + 131.1 * (if male) + 21.9 * (if CYP3A5*1/*1/*1/*3 /ABCB1CT/TT genotype) + 223.8 * (if *CYP3A5*3/*3 /ABCB1CC* genotype) + 216.1 (if *CYP3A5*3/*3 /ABCB1CT/TT* genotype).	49.4%	Age ($p = 0.006$, $R^2 = 3.6\%$) Sex ($p < 0.001$, $R^2 = 5.2\%$) Diabetes ($p = 0.005$, $R^2 = 4.5\%$) Donor type ($p = 0.004$, $R^2 = 3.7\%$) Time since transplantation ($p = 0.007$, $R^2 = 1.2\%$) *CYP3A5*/*ABCB1* genotype ($p < 0.001$, $R^2 = 31.1\%$)

R^2 gives the % variability explained by the independent variables in the multiple regression analysis model.

4.4 Discussion

4.4.1 Pharmacokinetics and Bioequivalence of Once- and Twice-Daily Tacrolimus Preparations in Stable Renal Transplant Patients.

Advagraf® showed a comparable pharmacokinetic profile to twice-daily tacrolimus, as assessed by comparing $AUC_{0–24}$ and C_{max} concentrations. The obtained data from 64 renal transplant recipients confirm that once-daily tacrolimus, Advagraf® is bioequivalent to twice-daily tacrolimus preparations in kidney transplant patients according to the FDA and EMA guidelines. At 90% CI values for the ratios of the geometric means of $AUC_{0–24}$ OD-Tac/TD-Tac (91% –102%) and of C_{max} OD-Tac/TD-Tac (84% –97%) were each within the stipulated range of 80–125%, which meets the FDA bioequivalence acceptable range applied for any bioequivalence study. These criteria allow no more than 20% higher or lower changes in $AUC_{0–24}$ and C_{max} ratios between the generic and the reference drugs (FDA., 2003). EMA guidelines which tighten the bioequivalence acceptable margin for AUC geometric mean ratio to 90.00 – 111.11% for narrow therapeutic index drugs including tacrolimus (EMA, 2010b) were also met. Our analysis showed that the Advagraf® was bioequivalent to twice-daily tacrolimus; however, it attained a lower C_{max} and a lower systemic profile of tacrolimus than twice-daily tacrolimus preparations. The results obtained supported a safe mg: mg conversion from twice a day to modified release tacrolimus, Advagraf®. These results are in agreement with the previously published data, which showed that the steady state tacrolimus pharmacokinetics of modified release tacrolimus is equivalent to Prograf® after mg: mg daily dose conversion in stable kidney transplant patients regardless of sex or race. The ratio of the OD-Tac/TD-Tac for $AUC_{0–24}$ was 95.0% with a 90% CI of 90.7% –99.4%. The ratio of the OD-Tac/TD-Tac for C_{max} was 88.2% with a 90% CI of 82.7% –94.0% (Alloway et al., 2005).This is contained within the 90% CI of 80% – 125%. Our results confirm previous results obtained from a steady-state study in stable kidney transplant recipients and found an equivalence of exposure at steady-state. Likewise, the 90% CI values for the ratios of geometric means of $AUC_{0–24}$ for days 14 and 21 once-daily tacrolimus versus days 1 and 7 twice-daily tacrolimus were 91% and 99%. They also demonstrate that kidney transplant recipients can be safely converted to

once-daily tacrolimus and tacrolimus concentration can be maintained using the same therapeutic monitoring and patient care techniques used for twice-daily tacrolimus (Alloway et al., 2007). Our results similar to the results obtained by van Hooff et al (2012).who found that tacrolimus $AUC_{0–24}$ for both formulations are comparable after mg: mg conversion from Prograf® to Advagraf®. At 90% CIs, the $AUC_{0–24}$ ratio was 92.9% (89.8%–96.0%), within the FDA bioequivalence acceptance range of 80%–125%. However, the treatment ratio (90% CI) for tacrolimus C_{max} was 73.2% (67.7%–78.7%), outside the range. Our results are in agreement with the $AUC_{0–24}$ ratio results obtained from once-daily tacrolimus PK study in paediatric kidney recipients. The ratio of the geometric means for $AUC_{0–24}$ was 90.8 %, with 90 % CI limits of 85.3%–96.7 %, falling within 80 % to 125 % the FDA bioequivalence limits. In contrast to our results, their C_{max} did not significantly differ between both formulations in the investigated study population. They demonstrated that the two formulations were bioequivalent (Lapeyraque et al., 2014). In contrast to our results, Min et al (2013) found that at steady state, the tacrolimus exposure was not equivalent between both formulations in paediatric transplant patients. The ratio of $lnAUC_{0–24}$ for OD-Tac/TD-Tac was 84.7 with 90% CI of 79.1%–90.8% after mg: mg conversion. lnC_{max} was also not equivalent; the 90% CIs was 70.7%–89.1%. Despite that the dose was adjusted, the ratio of $lnAUC_{0–24}$ was 87.8 (90% CI: 78.1%–98.9%) on day 28 of the study. In addition, there was a good correlation between $AUC_{0–24}$ and C_0 for immediate and modified release tacrolimus hence; it allows therapeutic monitoring via the same system as twice-daily Tacrolimus. Similar findings were noted in previous studies (Alloway et al., 2005, van Hooff et al., 2012).

*4.4.2 CYP3A5*3 and ABCB1 3435C>T Associations with Tacrolimus Preparations Pharmacokinetics and Dose Requirements.*

The present study assessed whether the well-defined association of the *CYP3A5*3* and *ABCB1* genotypes on the pharmacokinetics of immediate release tacrolimus also applies for the prolonged release preparation, Advagraf®.

Based on the analysis of the data obtained from 64 stable renal transplant recipients, it can be demonstrated that CYP3A5 expressers (*CYP3A5*1* allele carriers) required higher doses of tacrolimus than CYP3A5 non-expressers (*CYP3A5*3/*3*). In addition, tacrolimus dose-normalized C_0, C_{max} and AUC_{0-24} are significantly lower in CYP3A5 expresser patients than CYP3A5 non-expressers. This is in agreement with the previously published data which showed that the CYP3A5 expressers required a higher daily tacrolimus dose compared to non-expressers (Roy et al., 2006). Moreover, it was reported that tacrolimus pharmacokinetics were heavily dependent on CYP3A5 allelic status. Subjects carrying at least one functional *CYP3A5*1* allele (CYP3A5 expressers) metabolize tacrolimus more rapidly and require higher doses to reach target concentrations (MacPhee et al., 2002). Our findings confirmed the results obtained from a previous study in Japanese renal transplant recipients showing that the dose-adjusted C_{max} (µg/L/mg/kg), AUC_{0-12} (µg*h/L per mg/kg) and tacrolimus trough blood concentrations in *CYP3A5*1* carriers had a significantly lower value than the *3/*3* carriers, though the daily dose of tacrolimus mg/kg/day was significantly higher (Tsuchiya et al., 2004). Our results are in conformity with these studies. This is in agreement with the fact that the *CYP3A5*3* allele decreases CYP3A5 production and results in the loss of hepatic CYP3A5 activity (Hustert et al., 2001, Kuehl et al., 2001).

CYP3A5 could be useful to predict the optimal tacrolimus dose prior to transplantation. A recent study showed that kidney transplant recipients using tacrolimus doses according to the CYP3A5 genotype reached the target C_0 significantly earlier than recipients used a standard regimen (Thervet et al., 2010). Although more patients were within the desired tacrolimus target range early after transplantation, a considerable proportion of patients still did not have tacrolimus C_0 levels within the target range

indicating that *CYP3A5* genotype alone is unlikely to be sufficient for successful individualisation of initial tacrolimus dose.

With regards to *ABCB1 3435C>T*, we found that the patients with the *ABCB1 3435 CC* allele have a significantly higher tacrolimus dose mg/kg/day and lower tacrolimus dose-adjusted $AUC_{0–24}$, dose-adjusted C_{max} and dose-adjusted trough levels post transplantation compared to those with *ABCB1 T* variant homozygotes. Similarly, some studies have reported a correlation between *ABCB1 3435C>T* polymorphism and tacrolimus pharmacokinetics. Zheng et al. (2003) for instance, analysed tacrolimus pharmacokinetics in relation to *ABCB1 3435C>T* polymorphisms and found a significant association between the *ABCB1 3435C>T* polymorphisms and the dose of tacrolimus per day, showing that *CC* recipients require a higher dose than the *CT/TT* patients at 6 and 12 months after heart transplantation. Similarly, Patients with *C/C* homozygotes of *ABCB1 3435C>T* showed significantly lower dose-adjusted tacrolimus trough concentrations compared with *ABCB1 CT/TT* variant carriers in liver transplant recipients (Yu et al., 2011). In contrast, other studies have reported a weak correlation with *ABCB1 3435C>T* polymorphism and high tacrolimus blood concentrations (Diaz-Molina et al., 2012, Fredericks et al., 2006, Singh et al., 2011), whereas others found no effect (Jun et al., 2009, Quteineh et al., 2008). Other studies showed that there was no association of any tacrolimus parameter, such as tacrolimus dose/kg/day, dose-adjusted $AUC_{0–12}$, dose-adjusted C_{max} and trough concentration with *ABCB1 CC* and *CT/TT* genotype subgroups, indicating that *ABCB1 3435C>T* polymorphism was not an important factor in tacrolimus pharmacokinetics (Rong et al., 2010, Tada et al., 2005). Furthermore, Goto et al. (2002) found that none of the *ABCB1 3435C>T* variants studied showed a significant effect on tacrolimus concentration/dose ratio in the first week after transplantation.

After categorizing the renal transplant patients in different groups based on their *CYP3A5*3* and *ABCB1 3435C>T* genotype, no significant difference was observed in *CYP3A5*3* carriers with any of *ABCB1 3435C>T* variant alleles. However, significant differences in tacrolimus pharmacokinetic parameters were evident between *ABCB1 3435C>T* polymorphisms in *CYP3A5*1* allele carriers. This suggests that CYP3A5

expression had a major influence on tacrolimus exposure and that *ABCB1 3435C>T* polymorphism was an important factor in tacrolimus pharmacokinetics particularly in case of CYP3A5 expressers. In contrast to our results, a minor association was found between the *ABCB1 3435C>T* polymorphisms and tacrolimus blood concentration in CYP3A5 non-expressers suggesting that the variation in tacrolimus oral bioavailability is primarily produced by heterogeneity in the level of intestinal P-glycoprotein expression (MacPhee et al., 2002). Some studies are in agreement with our findings showing no significant differences in tacrolimus bioavailability between the *ABCB1 CC* and the *CT/TT* genotype subgroups in the non-expresser group (Rong et al., 2010, Tada et al., 2005). Likewise, these studies have reported the same outcome between *ABCB1 3435C>T* variants and *CYP3A5* expressers which contrast with our findings. Furthermore, another study found that upon evaluating the 2 genotypes for *CYP3A5*3* and *ABCB1 3435C>T* in combination, significant differences in tacrolimus Concentration/Dose ratios for the various groups reflected mainly the CYP3A5 polymorphism (Loh et al., 2008). This is in agreement with our finding.

The role of *CYP3A5*3* and *ABCB1 3435C>T* genotypes in metabolism and dose requirements of Advagraf® in renal transplant patients is currently unknown. It was reported that expression of CYP3A decreases and expression of P-gp increases along the length of the small intestine (Zhang and Benet, 2001). Theoretically, it cannot be assumed that the genetic factors known to influence the pharmacokinetics of the Prograf® preparation of tacrolimus will apply to the prolonged release preparation, Advagraf®, which is absorbed more distally. Contrary to expectations, however, the influence of the *CYP3A5*3* and *ABCB1 3435C>T* genotype on tacrolimus exposure is the same for the prolonged release preparation Advagraf® as for immediate release preparation of tacrolimus. This means that any pharmacogenetic algorithms based on these genotypes can be applied to both preparations.

When data were analysed based on the presence or absence of diabetes, we did not find any statistically significant differences ($p = 0.056$). However, another recent study showed that diabetic patients have significantly higher dose adjusted tacrolimus levels compared to non-diabetic patients (Chitnis et al., 2013) and our study is likely to have

been statistically underpowered to pick this up. Age appeared to be associated with tacrolimus dosing requirements. Our findings demonstrated that tacrolimus dosing requirements were higher in younger than older patients. This is in line with previous reports showed that age correlated significantly with tacrolimus dose in both adults (Kim et al., 2012) and paediatric patients (Gijsen et al., 2011). In addition, patient sex greatly affected tacrolimus dose requirements. Tacrolimus dose was significantly lower in males compared to females. This is in accordance with the results of a recent study identified male sex as a significant risk factor for being slower tacrolimus metabolizers (Stratta et al., 2012). Similarly, our results also confirm a minor role of the *ABCB1 3435C>T* variant allele. In contrast, *CYP3A5*3* allele is a key factor in the interpretation of tacrolimus blood concentrations and dose requirement. Furthermore, the haematocrit value was significantly associated with tacrolimus dose requirements, consistent with previous reports (de Jonge et al., 2012, Stratta et al., 2012). Previous report has described higher tacrolimus dose requirement in black patients (Macphee et al., 2005), which is consistent with our findings.

Finally, multiple regression analysis by stepwise selection identified the combined *CYP3A5*3* and *ABCB1 3435C>T* genotype, sex, age, haematocrit value, ethnicity, corticosteroids treatment and diabetic status as independent variables associated with tacrolimus dose requirements. We found that the combined *CYP3A5*3* and *ABCB1 3435C>T* genotype may explain 36.9% of the variability in tacrolimus dose requirements. Age, sex, haematocrit value, ethnicity, corticosteroids treatment and diabetic status may partially explain the gap in the variability in tacrolimus dose requirement that cannot be explained by genetic factors in renal transplant patients. Our findings suggest that taking all these aforementioned factors into consideration may account for 59.9% of the between-individual variability in tacrolimus dose requirements. These findings may have potential clinical application for initiation and adjustment of tacrolimus therapy.

Chapter 5. Associations of *CYP3A4*22* and *POR*28* Genotypes with Pharmacokinetics of Immediate and Prolonged Release Tacrolimus Preparations.

This study is part of a larger pharmacokinetic study; detailed information on the study participants and procedure can be found in Chapter 4. The objectives of the study were:

1. To develop new methods for genotyping *CYP3A4*22* and *POR*28* SNPs using a LightCycler based technique.
2. To study the relationship between *CYP3A4*22* and *POR*28* SNPs and the pharmacokinetics of immediate release tacrolimus; Prograf® and Adoport® and prolonged release; Advagraf® within individual patients.

*5.1 Rapid Genotyping Methods for CYP3A4*22 and POR*28 Polymorphisms with Real-time Polymerase Chain Reaction Fluorescence Resonance Energy Transfer Assays.*

5.1.1 Introduction

The cytochrome P450 family (CYP450) represents a family of proteins that have an important role in first-pass metabolism. The most important cytochrome P450 isoforms responsible for drug metabolism are the CYP3A family and mainly present in tissues such as the gastrointestinal tract and liver. CYP3A enzymes are involved in the oxidative biotransformation of numerous clinically useful therapeutic agents (Wilkinson, 1996). Within this subfamily, CYP3A4 is the most abundant CYP enzyme and involved in the metabolism of 45–60% of all currently used drugs (Danielson, 2002). CYP3A4 activity shows 10- to 100-fold variation between individuals (Wang et al., 2011). Recently, a mutation associated with reduced CYP3A4 activity has been described: *CYP3A4*22* rs35599367C>T. The *CYP3A4*22* SNP was correlated with the dose requirements of some drugs, including statins and tacrolimus (Elens et al., 2011a, Wang et al., 2011).

Cytochrome P450 reductase also known as NADPH: P450 oxidoreductase (POR),a membrane-bound enzyme, serves as the electron donor to almost all cytochromes P450 (Jensen and Møller, 2010). It supplies electrons to all microsomal P450s for catalytic activity and the *POR*28* (rs1057868C>T) SNP is the most common sequence variant. It was reported that *POR*28* was associated with CYP3A activity (Oneda et al., 2009). Recently, some studies showed a significant association between *POR*28* and tacrolimus pharmacokinetics and dose requirement. CYP3A5 expressers carrying at least one *POR*28* allele have shown higher tacrolimus dose requirements than CYP3A5 expressers carrying no *POR*28* alleles (de Jonge et al., 2011). RT-PCR allows quantification of the target polymorphic DNA regions and genotyping of single nucleotide polymorphisms (SNPs) in one single PCR run. It facilitates a high speed PCR analysis and minimizes the risk of contamination during analysis.

5.1.2 Materials and Methods

Ethylenediaminetetraacetic acid anticoagulated whole blood samples were collected from 22 patients and stored at –20°C until DNA isolation. DNA was isolated from 200µl EDTA anticoagulated blood using QIAamp DNA Blood Mini Kit (QIAGEN®, West Sussex, UK). PCR was performed using LightCycler® 2.0 supplied from Roche® (Lewes, UK). The LightCycler FastStart DNA Master Hybridisation Probe kit was supplied from Roche® (West Sussex, UK). The primers and the fluorogenic probes used were designed and synthesised by TIB MOLBIOL (Berlin, Germany). Two primers and probes were received from TIB MOLBIOL for each SNP and several fluorescence resonance energy transfer (FRET) designs have been tested to get the best peak intensity and then Mg^{2+} concentration in the reaction mixture was also adjusted and optimized to get much better resolution.

Restriction Fragment Length Polymorphism Analysis of PCR-Amplified Fragments (PCR-RFLP) for the *CYP3A4*22* and *POR*28* polymorphisms was used to confirm the results obtained by the real-time PCR FRET assays. In order to validate FRET genotyping methods, samples of different *CYP3A4*22* and *POR*28* polymorphisms were sequenced. PCR was performed using PTC 100 Peltier thermal cycler (Bio-Rad

Laboratories Ltd, Hertfordshire, UK) and a Mastercycler pro S vapo.protect Thermal Cycler (Eppendorf, USA). PCR products were electrophoresed on a 5% agarose gel (Sigma Aldrich, UK) containing 0.3 mg/l ethidium bromide at 75V for 1 hour and examined under ultraviolet illumination. The PCR products were sequenced according to a direct sequence procedure performed with the capillary sequencer ABI 3130xL Genetic Analyser (Applied Biosystems, Warrington, UK) using BigDye® Terminator v3. 1 Cycle Sequencing Kit was supplied from Applied Biosystems® (Warrington, UK).

*5.1.2.1 CYP3A4*22 and POR*28 Genotyping with Real-time PCR*

*5.1.2.1.1 CYP3A4*22 C >T Genotyping*

In this method we used two primers, forward and reverse and one single probe. The sequence of the forward primer was TgATAgTgggTCTCTgTCTTCC and of the reverse primer AgCCACAgACTTTCAgATCTACTAg while the sequence of the probe was CTACACTgggTgTgA XI TggAgACACT—PH. The reaction mixture made up of 0.5 µM of each primer and 0.3 µM of the probe. 2 µL of DNA samples was added to each reaction. Three control samples were included in each LightCycler run, two positive controls and one negative control (Deionized water). The samples were loaded into composite glass capillaries (Roche Molecular Biochemicals) containing 18µL of the reaction mixture, centrifuged, and placed in the LightCycler sample carousel. The program was designed using 60°C annealing temperature. After an initial denaturation step at 95°C for 10.0 min, amplification was performed using 45 cycles of denaturation (95°C for 5 s), annealing (55°C for 10 s), and extension (72°C for 20 s). After amplification was complete, a final melting curve was recorded by annealing at 40°C for 20s, followed by an increase in temperature at a rate of 0.1°C/s, with continual fluorescence detection up to 85°C. The florescence display was at F1 mode for fluorescence display. Melting curves were converted into melting peaks using the LightCycler Software by plotting the negative derivatives of fluorescence against temperature (- dF/dT).

*5.1.2.1.2 POR*28 C >T Genotyping*

The sequence of the forward primer was CACAggTCCACCCCAACTCT and of the reverse primer CAggCggAACTgggACTT while the sequence of the probe was ggAgCCTgCCggggA XI AACg--PH. The method used for *POR*28* was the same as *CYP3A4*22*. The reaction mixture consisted of 0.1 µM (2 pmoL in 20 µL) of the forward primer with 0.5 µM (10 pmoL) of the reverse primer and 0.5 µM probe. Three control samples were included in each LightCycler run, two positive controls and one negative control (Deionized water). 2 µL of DNA samples were loaded into composite glass capillaries (Roche Molecular Biochemicals) containing 18µL of the reaction mixture, centrifuged, and placed in the LightCycler sample carousel. The PCR protocol included the following steps: pre- incubation phase at 95°C for 10 min to activate the Taq polymerase enzyme, followed by amplification phase, which includes 45 cycles of denaturation (95°C, 10s), annealing (55°C, 5 s) and extension (72°C, 15 s). Then this was followed by melting curve analysis stage, which involved annealing at 60°C for 50s, followed by an increase in temperature at a rate of 0.1C/s, with continual fluorescence detection up to 95°C. The florescence display was at F1 mode for fluorescence display. Melting curves were converted into melting peaks using the LightCycler Software by plotting the negative derivatives of fluorescence against temperature (- dF/dT).

*5.1.2.2 CYP3A4*22 and POR*28 Genotyping with DNA Sequencing*

The PCR products of twenty two patients' DNA samples were sequenced by the capillary sequencer ABI 3130xL Genetic Analyser® (Applied Biosystems, Warrington, UK) using BigDye® Terminator v3. 1 Cycle Sequencing Kit supplied from Applied Biosystems® (Warrington, UK). Electropherograms of each PCR fragment were obtained containing the SNPs of interest. DNA samples were amplified using the same primers used with the LightCycler for those four SNPs. These samples were analysed to confirm the genotypes obtained using the LightCycler. Each sequence of PCR fragment was confirmed in both directions, forward and reverse directions. The genotyping assays consisted of the following five steps.

5.1.2.2.1 PCR Amplification

Each PCR was performed in a reaction volume of 20µL containing DNA master mix, $MgCl_2$, forward and reverse primers, with the same protocol mentioned in its LightCycler method, and 2µL of genomic DNA. The DNA master mix (Roche®, UK) consisted of Taq DNA polymerase, reaction buffer, dNTP mix (with dUTP instead of dTTP) and 10mM $MgCl_2$. The PCR cycle protocol was done for the 2 SNPs and consists of the following steps, initial denaturation step at 95°C for 10.0 min, amplification was performed using 40 cycles of denaturation (95°C for 10 s), annealing (65°C for 10 s), and extension (72°C for 20 s), followed by a final extension at 72°C for 10.0 min.

In order to check whether the PCR generated the anticipated DNA fragment, agarose gel electrophoresis was employed. PCR products were electrophoresed on a 5% agarose gel (Sigma Aldrich, UK) containing 0.3 mg/l ethidium bromide covered with TBE buffer, buffer solution containing a mixture of Tris base, boric acid and EDTA, at 75V for 1 hour and examined under ultraviolet illumination. TBE buffer was obtained from Sigma Aldrich, UK.

5.1.2.2.2 Shrimp Alkaline Phosphatase (SAP) and Exonuclease I Treatment.

In order to remove unincorporated deoxynucleotide triphosphates and primers from the PCR amplification step, the PCR products were treated with Fermantas® shrimp alkaline phosphatase and exonuclease I (Thermo Scientific®, UK). A 3.2 µL aliquot of a mixture of shrimp alkaline phosphatase (240 µL of 1unit/µL), exonuclease I (12 µL of 20 unit/µL) and diluted SAP buffer (120 µL) was added to the PCR product and the mixture was incubated at 37°C for 120 min, followed by an incubation at 85°C for 20 min (to denaturate the enzymes), in the pro S vapo.protect Thermal Cycler. The treated PCR products were then kept at -20°C until use.

5.1.2.2.3 DNA Sequencing using Dye-Terminator Cycle-Sequencing.

To sequence the PCR product, BigDye-terminator cycle-sequencing kit (Applied Biosystems, UK) was used. In the cycle-sequencing process, 2µL of the PCR template was added to 1 µL of the primer (forward or reverse), 0.5 µL of BigDye mix to each tube, and was brought to a final volume of 10 µL with dH_2O (6.5µL). ABI-supplied BigDye mix consisted of Taq DNA polymerase, dNTPs (deoxy nucleotide triphosphates) in large concentration and ddNTPs (dideoxy nucleotide triphosphates) with fluorescent dyes in low concentration. In this reaction only one primer is used so we did one forward and one reverse reaction. After an initial denaturation step at 96°C for 5.0 min, amplification was performed using 30 cycles of denaturation (96°C for 10 s), annealing (57°C for 5 s), and extension (60°C for 4.0 min), followed by termination at 4°C for 10.0 min.

5.1.2.2.4 Reaction Clean Up

Sequencing reactions need to be cleaned up prior to injection on the ABI sequencer in order to remove salts from the reaction buffers, unincorporated nucleotides, and dye terminators that can interfere with electrophoretic dynamics and laser function. Such cleaning was done by filtering through the sephadex column. The sephadex mixture was prepared using 10g of sephadex beads (Sigma Aldrich, UK) and 150 mL distilled water, the solution was microwaved with a normal setting for 1 minute, and then the solution was allowed to cool using magnetic stirrer and spinner for a few hours. Sephadex mixture (500 µL) was loaded on each tube and spun at 1000 x g to get rid of excess water. Then the sephadex columns were placed on top of a clean PCR plate. Following this step, 10 µL of the mixture was loaded directly into the centre of each sephadex well and it was spun again at 1000 x g to obtain the filtered sample. The filtrate was then left in the PCR plate, and was placed in a PCR machine at 90°C till dryness. It was then reconstituted with 12 µL of formamide (Sigma Aldrich, UK), and the plate was covered with an adhesive PCR film (Thermo Scientific, UK), heated at 95°C for 5.0 min, and fast frozen at -20°C until use.

5.1.2.2.5 Genotype Determination on the Sequencer

The labelled sequencing products were sequenced on the Applied Biosystems 3130xL Genetic Analyser. After the sequencing reactions, the plate was placed in the sequence analyser and linked with standard method. The first run took 45 min and the run after that took 30 min. The created raw files were analysed with sequencing analysis 5.3.1 program to create the electropherograms. These electropherograms were visualized with FinchTV program to obtain the genotyping.

5.1.2.3 Statistical Analysis

The genotype distribution for each polymorphism was assessed for deviation from Hardy–Weinberg equilibrium, and differences in genotype frequency and in allele frequency between the groups were assessed using the Chi-squared test.

5.1.3 Results

For CYP3A4 rs35599367C>T polymorphism, the melting point of the sensor probe was 61°C when hybridised to *CYP3A4*22 CC* genotype. While the heterozygote polymorphism *CYP3A4*22CT* presented two melting peaks, at 61ºC and 66ºC **(Figure 34 A)**. In our study population, there were no *CYP3A4*22 TT* homozygotes which would be anticipated to have a melting peak at 66ºC. For the *POR*28* polymorphism, the melting peak for *POR*28 CC* homozygotes was at 62ºC, whereas melting peaks for *POR*28 TT* homozygotes were at approximately 50ºC. While in the heterozygote polymorphism *POR*28 CT*, both melting peaks appeared (**Figure 34 B**). Twenty PCR products were randomly selected from the genotype melting profiles for DNA sequencing conformation. *CYP3A4*22* and *POR*28* allelic variants and results obtained by real time PCR were confirmed by sequencing these genes. 100% concordance was obtained between the genotype and SNPs assessed by LightCycler. The DNA sequencing results for *CYP3A4*22* and *POR*28* are displayed in (**Figure 35**).

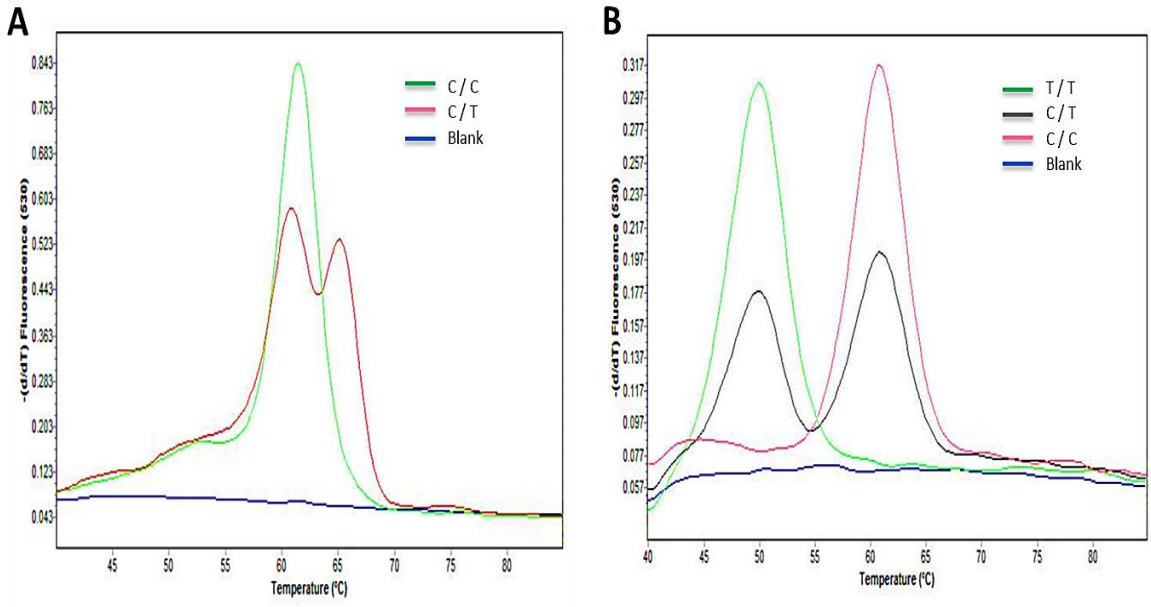


Figure 34: Derivative Melting Curve Plots for *CYP3A4*22* and *POR*28* Genotyping using Specific Primers and Probes. A. The Derivative Melting Curve for the *CYP3A4*22* Genotypes. Samples with the Homozygous *C* Allele (; Tm 61°C) and Heterozygous *C/T* genotype (; Tm 61°C and 66°C). B. The Derivative Melting Curve for the *POR*28* Genotypes. Samples with the Homozygous *T* Allele (; Tm 50°C); Homozygous *C* Allele (; Tm 62°C) and Heterozygous *C/T* Genotype (; Tm 50 °C and 62°C). The Melting Curve of a No Template Control in Both Assays ( ).

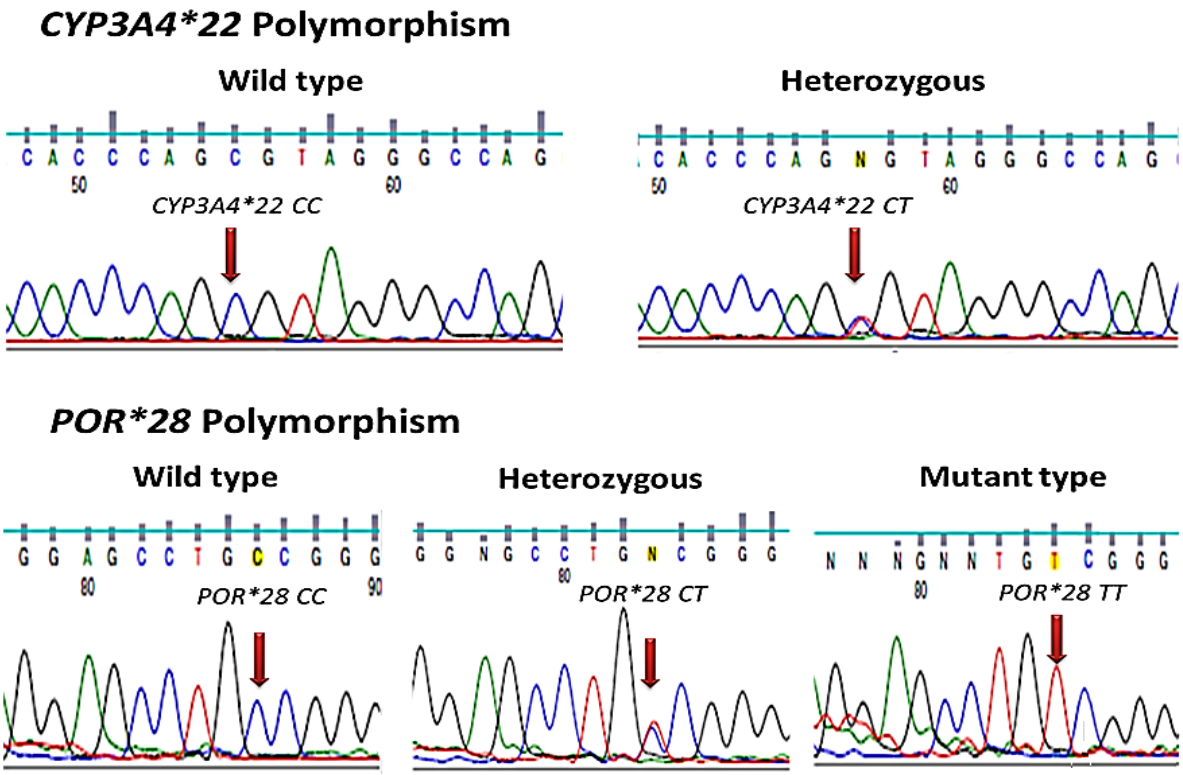


Figure 35: Sequencing of Representative Samples of *CYP3A4*22* and *POR*28* SNPs Showing the Sequencing Results of the Representative Wild Type, Heterozygous and Homozygous Mutants of *CYP3A4*22* and *POR*28* Polymorphisms.

*CYP3A4*22* and *POR*28* genotypes and allele frequencies are presented in **Table 28**. The genotype frequencies of the recipients were consistent with Hardy-Weinberg equilibrium ($p > 0.05$).

Table 28: Genotype Frequencies for *CYP3A4*22* and *POR*28* in Renal Transplant Patients

Gene	Variant	Genotype	n (%)	Genotype frequency (%)	Allele frequency
CYP3A4*22	c.522-191 C>T	*C/C*	59	92.2%	C = 0.96
		C/T	5	7.8%	T = 0.04
		T/T	0	0.0%	
POR*28	A503V	*C/C*	30	46.9%	**1* = 0.71
		C/T	31	48.4%	**3* = 0.29
		T/T	3	4.7%	

5.1.4 Discussion and Conclusions

In this study, we developed new methods for genotyping *CYP3A4*22* and *POR*28* SNPs uses a LightCycler based technique. Recently, some studies explored the relationship between *CYP3A4*22* and *POR*28* alleles and tacrolimus exposure (Elens et al., 2011a, Elens et al., 2013a). These studies demonstrated that there is an effect of these genotypes on daily doses required for renal transplant patients. In our study, we determined the genotypes of *CYP3A4*22* and *POR*28* using the LightCycler method for 22 patients. The genotypes of patients' samples were conducted using DNA sequence analysis. There was 100% concordance between the genotypes of the samples determined by sequencing and the genotypes determined by the LightCycler method. We present here a single-step method for homogeneous genotyping of the polymorphisms of the *CYP3A4*22* and *POR*28* genes that combines rapid-cycle PCR and fluorescent probe melting point analysis. Capillary PCR using the LightCycler is a reliable, convenient and simple method for genotyping *CYP3A4*22* and *POR*28* polymorphisms. It shows excellent correlation with genotype determination by DNA sequencing. This is a rapid method and because of its robustness, speed, and accuracy, this assay is suitable to determine the *CYP3A4*22* and *POR*28* polymorphisms in small and large number of samples.

5.2 CYP3A4*22 and POR*28 Genotype Associations with Tacrolimus Pharmacokinetics and Dose Requirements in Twice-Daily Tacrolimus and Advagraf®.

5.2.1 Objective of the Study:

The aim of this study is investigating the relationship between *CYP3A4*22* and *POR*28* SNPs and the pharmacokinetics of immediate release tacrolimus, Prograf® and Adoport® and prolonged release, Advagraf® within individual patients.

5.2.2 Materials and Methods

5.2.2.1 Study Design

Tacrolimus pharmacokinetic data obtained from the previous study for 64 stable kidney transplant recipients were used in this study. Patients were genotyped for *CYP3A4*22 C >T* and *POR*28 C >T* polymorphisms using the same DNA samples extracted for *CYP3A5*3* and *ABCB1 3435* genotyping. The C and T alleles of *POR*28* variant and in intron 6 of *CYP3A4*22* variant were identified by the new developed RT-PCR using LightCycler as described previously. For each gene, subjects were divided into two groups according to their genotype. For *CYP3A4*22,* patients were divided into *CYP3A4*22 CC* and *CYP3A4*22 CT/TT* genotype groups and for *POR*28,* it was *POR*28 CC* and *POR*28 CT/TT* genotype groups.

5.2.2.2 Pharmacokinetic and Statistical Analysis

Statistical analysis was performed using Minitab statistical software (Minitab 17). Tacrolimus individual pharmacokinetic parameters were determined for each genotype group. The log-transformed data were analysed using analysis of variance (ANOVA) with factors for genotype group and treatment.

5.2.3 Results

5.2.3.1 Patient Characteristics

The characteristics of the 64 patients included in the study are reported in **Table 29**. According to *CYP3A4*22* genotypes, 59 patients were homozygous for the C wild-type allele, while five patients carried one copy of the T-variant allele for the CYP3A4 intron 6 SNP. This corresponds to a calculated allelic frequency of 7.8% in our study population. No significant differences were found between the *CYP3A4* genotype groups except for the patients' age ($p = 0.014$) and ethnicity ($p = 0.015$). All *CYP3A4*22 CT* carriers were CYP3A5 non-expressers and they were all from White ethnic background.

Concerning *POR*28* genotypes, 30 patients had the CC genotype, 31 patients had CT genotype and three were homozygous TT. We found no significant difference between the *POR*28* genotype groups concerning the patients' age ($p = 0.123$) and sex ($p = 0.387$; **Table 29**).

Table 29: Patients Demographic Characteristics and Immunosuppression Therapy.

Characteristic	*CYP3A4*22 CC* carriers (n = 59)	*CYP3A4*22 CT* carriers (n = 5)	*POR*28 CC* carriers (n = 30)	*POR*28 CT/TT* carriers CT (n = 31) & TT (n = 3)
Sex				
Male/female	39/20	4/1	19/11	24/10
Age (y), mean (SD)	55.4 ± 12.2	45.2 ± 16.7	56.4 ± 13.3	52.9 ± 12.2
Race, n (%)				
White	34 (58%)	5 (100%)	19 (63%)	20 (59%)
Black	12 (20%)	-	6 (20%)	6 (18%)
Asian	13 (22%)	-	5 (17%)	8 (24%)
Body weight (kg), mean (SD)	76.5 ± 15.2	75.1 ± 16.5	77.5 ± 16.0	75.4 ± 14.6
Height (cm), mean (SD)	169.7 ± 8.5	175.5 ± 8.1	170.9 ± 10.2	169.4 ± 6.7
Diabetes, n (%)	14 (24%)	1 (20%)	9 (30%)	6 (18%)
Time since transplantation (years)				
Mean (SD)	4.2 ± 4.6	3.4 ± 5.0	4.0 ± 4.1	4.2 ± 5.1
Median	2.2	1.3	2.2	1.8
Donor type, n				
Living / Deceased	22/37	4/1	12/18	14/20
Serum creatinine (µmol/L)	125.3 ± 41.5	114.6 ± 11.5	120.1 ± 41.4	128.5 ± 38.8
Serum albumin(g/L)	38.8 ± 2.9	38.5 ± 5.2	38.8 ± 3.1	38.8 ± 3.1
Haemoglobin, (g/L)	128.9 ± 22	137.8 ± 27.3	129.4 ± 15.8	129.8 ± 27.0
Immunosuppression therapy				
Tacrolimus, n (%)				
Prograf®/ Adoport®	44 (75%) / 15 (25%)	3 (60%) / 2 (40%)	20 (67%) / 10 (33%)	27 (79%) / 7 (21%)
Corticosteroids, n (%)	36 (61.0%)	3 (60%)	17 (57%)	22 (65%)
Azathioprine, n (%)	12 (20%)	2 (40%)	7 (23%)	7 (21%)
Mycophenolate, n (%)	19 (32%)	-	9 (30%)	10 (29%)
***CYP3A5* Polymorphism**				
*CYP3A5 *1/*1/ *1/*3*	30 (12/18)	-	17 (6/11)	13 (6/7)
*CYP3A5 *3/*3*	29	5	13	21

*5.2.3.2 CYP3A4*22 & Tacrolimus Disposition*

The analysis has been done on 128 24-h PK profiles obtained from 64 patients with pooling of data for OD-Tac and TD-Tac. Tacrolimus blood concentrations C_{max}, AUC_{0-24} and C_0 showed a log-normal distribution, and the data were therefore log-transformed before analysis. As shown in **Table 30**, a significant difference in dose-normalized C_0 was observed according to the patients *CYP3A4*22* allelic status. Patients carrying a T variant allele had 1.8 fold higher dose-normalized C_0 compared to homozygous CC allele carriers (P= 0.006). The dose-normalized C_{max} was higher for the T variant carriers than for CC patients: 37.2 ±15.4µg/L vs 25.8 ±13.9µg/L (P=0.015). Moreover, a significant difference was observed in AUC_{0-24} between the two genotypes; *CYP3A4*22CC* and *CYP3A4*22CT* carriers (329.9 ± 198.6 vs. 562.0 ± 313.3, p = 0.006). Despite that the T allele carriers required significantly lower tacrolimus doses than CC patients to reach this C_0. The mean daily dose requirement of tacrolimus per body weight was 38.2% lower for T-variant allele carriers compared to CC allele carriers (p = 0.006; see **Figure 36**).

Table 30: Tacrolimus Dose-Normalized PK Parameters According to *CYP3A4*22* Genotypes for the Whole Data of Once-and Twice-Daily Tacrolimus.

PK-parameter	*CYP3A4*22 CC* (n= 118)	*CYP3A4*22 CT* (n= 10)	P-value
Dose (mg/Kg/day)	0.08 ± 0.05	0.05 ± 0.04	<0.01
C_{max} (µg/L/mg/Kg)	25.8 ± 13.9	37.2 ± 15.4	<0.05
AUC_{0-24} (µg*h/L/mg/Kg)	329.9 ± 198.6	562.0 ± 313.3	<0.01
C_0 (µg/L/mg/Kg)	10.6 ± 7.4	19.5 ± 13.0	<0.01

Values were compared using ANOVA (General linear model).

Upon comparing the influence of *CYP3A4*22* polymorphism on both tacrolimus formulations, we found no association between *CYP3A4*22* genotypes and tacrolimus dose-normalized $AUC_{0–24}$, C_{max} and C_0 (p > 0.05), see **Table 31** and **Figure 37**. In *CYP3A4*22 CC* group, the ratio of OD-Tac/TD-Tac $AUC_{0–24}$ was 98% and its 90 % CI (82%–117%). In addition, the ratio of the OD-Tac/TD-Tac for C_{max} was 90% (90% CI

79% –103%), falling outside 80% to 125% bioequivalence limits. Therefore, the two formulations were not bioequivalent. According to EMA guidelines, the confidence interval of the AUC_{0-24} ratio was outside the bioequivalence margin (90-111%). Therefore both formulations were not bioequivalent. These data are summarized in **Table 32.**

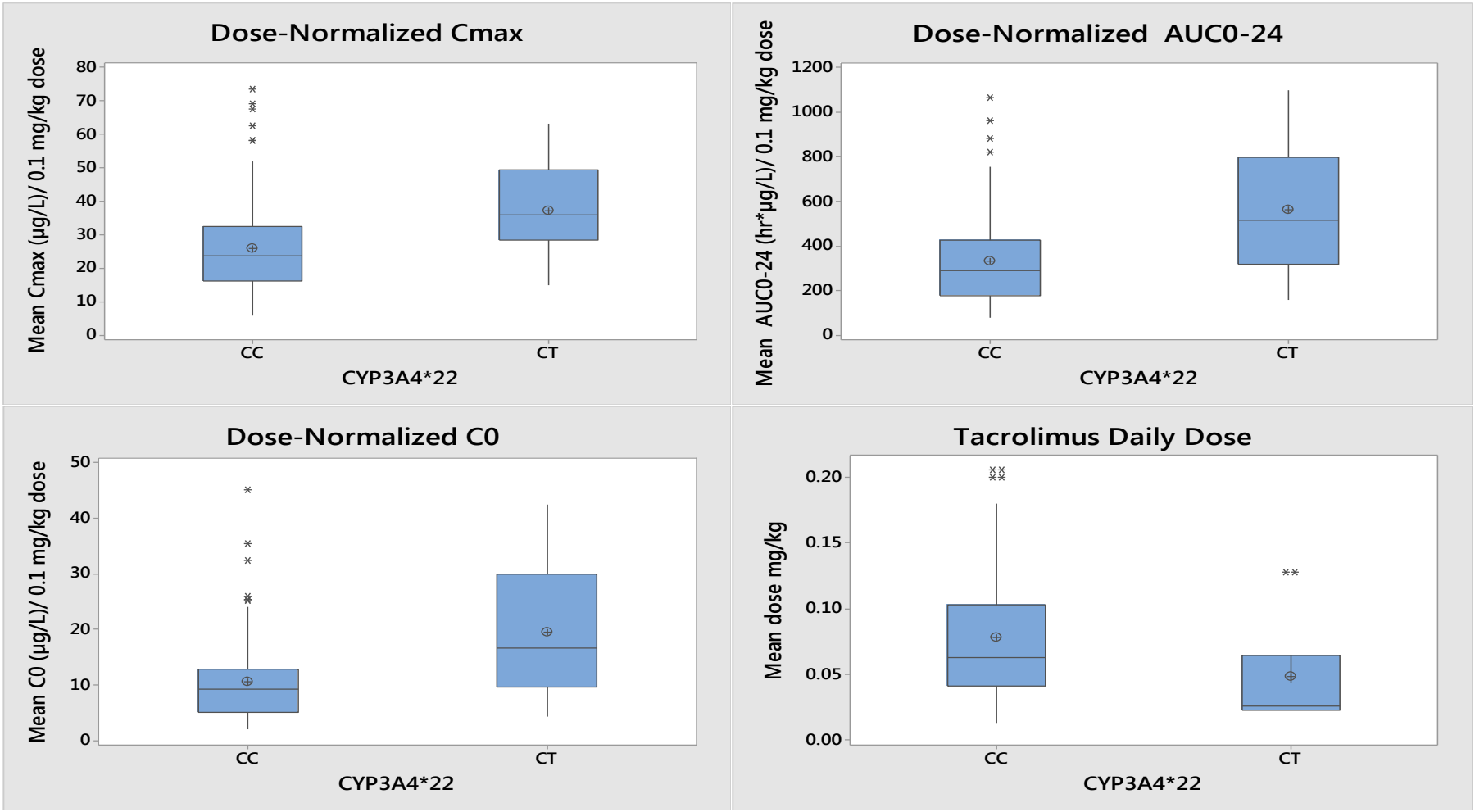


Figure 36: *CYP3A4*22* Genotype Associations with Tacrolimus Dose and Dose-Normalized Pharmacokinetic Parameters for the Whole Data of Once-and Twice-Daily Tacrolimus. Boxes represent the interquartile range, line in the box represents the median value, symbol in the box represents the mean value, outer lines represent the minimal and maximal value and the values beyond the whiskers (asterisks) are outliers.

Table 31: *CYP3A4*22* Polymorphism Relationship with Dose-Normalized Tacrolimus Pharmacokinetic Parameters in Different Tacrolimus Formulation (Twice-Daily Tacrolimus; TD-Tac, and Advagraf®).

PK-parameter	*CYP3A4*22 CC* (n= 59)			*CYP3A4*22 CT* (n= 5)		
	TD-Tac	**Advagraf®**	**P-value**	**TD-Tac**	**Advagraf®**	**P-value**
Dose (mg/Kg/day)	0.08 ± 0.05	0.08 ± 0.05		0.048 ± 0.045	0.048 ± 0.045	
AUC_{0-24} (µg*h/L/mg/Kg)	340 ± 223	320 ± 173	0.85	582 ± 351	542 ± 311	0.92
C_{max} (µg/L/mg/Kg)	27.5 ± 15.8	24.2 ± 11.6	0.37	39.2 ± 17.6	35.3 ± 14.6	0.79
C_0 (µg/L/mg/Kg)	11.4 ± 8.8	9.7 ± 5.7	0.45	20.9 ± 14.5	18.2 ± 12.8	0.81

Values were compared using ANOVA (General linear model).

Table 32: Bioequivalence Statistics for $AUC_{0–24}$ and C_{max} for Twice-Daily Tacrolimus (TD-Tac) and Once-Daily Tacrolimus (OD-Tac) in *CYP3A4*22 CC* Carriers.

Parameter	*CYP3A4*22 CC*	
	Ratio of geometric means (%)	**90% CI**
$AUC_{0–24}$	98%	(82% – 117%)
C_{max}	90%	(79% – 103%)

90% CI for geometric mean are based on the ANOVA model (General linear model).

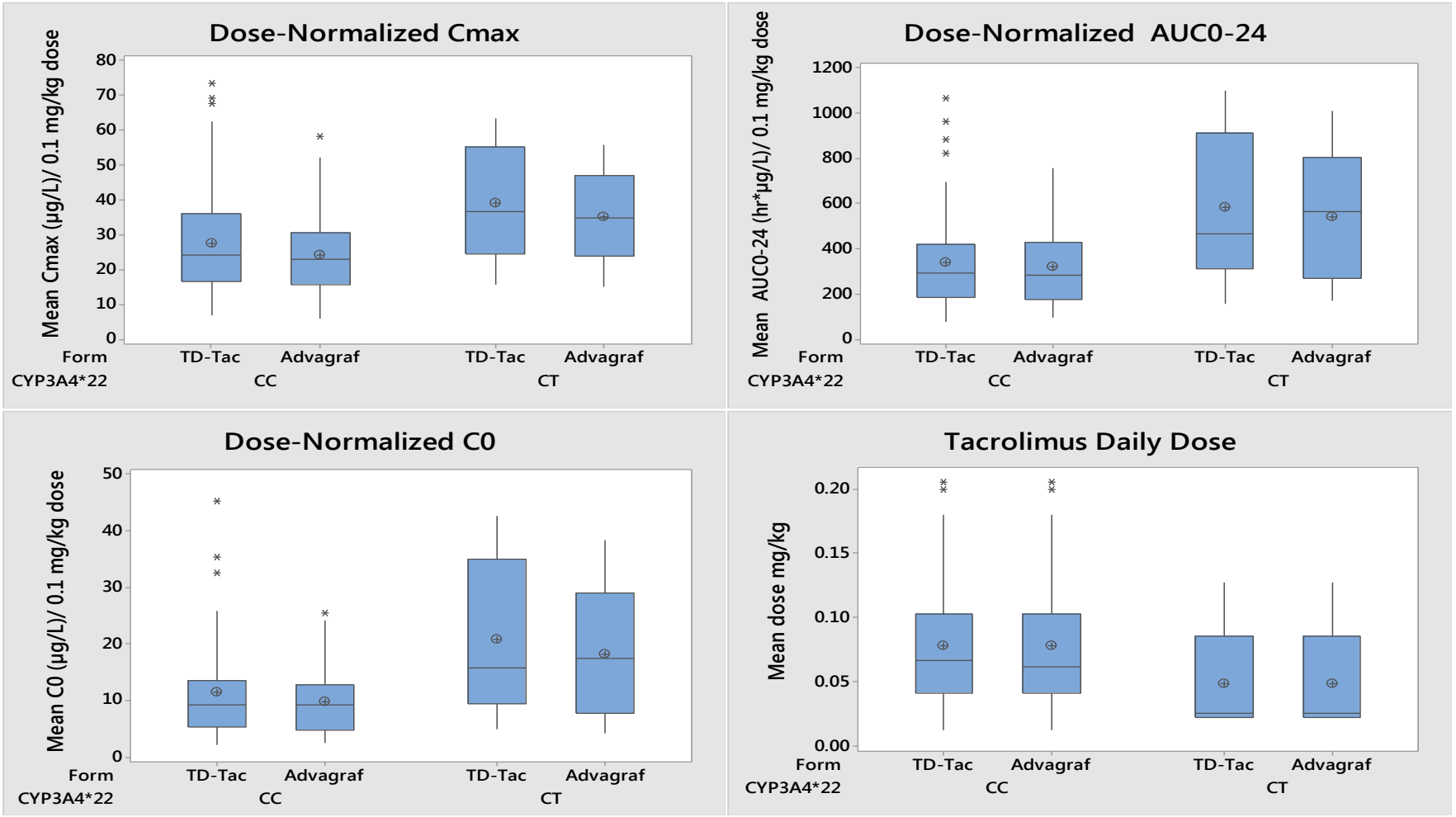


Figure 37: *CYP3A4*22* Genotype and Tacrolimus Pharmacokinetic Parameters of Twice-daily Tacrolimus (TD-Tac) and Advagraf®. Boxes represent the interquartile range, line in the box represents the median value, symbol in the box represents the mean value, outer lines represent the minimal and maximal value and the values beyond the whiskers (asterisks) are outliers.

*5.2.3.3 CYP3A5*3-CYP3A4*22 Combined Genotypes & Tacrolimus Disposition*

To investigate the combined associations between *CYP3A5*3* and *CYP3A4*22* genotype and dose-normalized tacrolimus PK parameters, patients were divided with respect to CYP3A5 expressers (*CYP3A5*1* allele carriers) or CYP3A5 non-expressers (*CYP3A5*3/*3* carriers) and *CYP3A4*22* high activity (*CYP3A4*22 CC* carriers) or medium-activity (*CYP3A4*22 CT* carriers) groups (Elens et al., 2011a). Three genotype groups were identified in our study population: extensive CYP3A metabolizers (*CYP3A5*1* allele carriers /*CYP3A4*22CC*, n=30), intermediate CYP3A metabolizers (*CYP3A5*3/*3* /*CYP3A4*22CC*, n=29) and poor CYP3A metabolizers (*CYP3A5*3/*3* /*CYP3A4*22CT*, n=5). The analysis has been done on 128 24-h PK profiles obtained from 64 patients with data for TD-Tac and OD-Tac pooled.

Extensive CYP3A metabolizers required a significantly higher daily dose of tacrolimus compared to both the intermediate CYP3A metabolizers (P< 0.001) and poor CYP3A metabolizers groups (P< 0.001). However, the difference between the intermediate metabolizers and poor metabolizers was not statistically significant (p= 0.2). On the other hand, poor CYP3A metabolizers showed 95% higher dose-normalized tacrolimus C_{max} compared to extensive metabolizers (p < 0.0001) and 13% higher dose-normalized tacrolimus C_{max} compared to intermediate metabolizers (p= 0.4). The same trend was observed for tacrolimus dose-normalized $AUC_{0\text{-}24}$ and C_0. The intermediate CYP3A metabolizers had a slight, but not statistically significant, decrease in dose-normalized $AUC_{0\text{-}24}$ (p= 0.3) and C_0 (p= 0.4) compared to poor CYP3A metabolizers, see **Figure 38 & Table 33**.

No significant differences were found in tacrolimus dose and pharmacokinetic parameters between tacrolimus formulations in each group of the combined CYP3A genotypes (**Figure 39; Table 34)**. The ratio of means (90% CI) of $AUC_{0–24}$ for OD-Tac versus TD-Tac was as follows: extensive CYP3A metabolizers, 101% (88% –116%); Intermediate CYP3A metabolizers, 92% (81% –104%). Whereas the ratio of means (90% CI) of C_{max} for OD-Tac versus TD-Tac was as follows: extensive CYP3A metabolizers, 93% (77% –111%); Intermediate CYP3A metabolizers, 88% (74% –

104%). These ratios did not achieve bioequivalence limits of 80–125%. Based on C_{max} results, TD-Tac and OD-Tac are no longer bioequivalent on a 1:1 conversion (**Table 35**). According to EMA guidelines, the confidence intervals of the AUC_{0-24} ratios were outside the bioequivalence margin (90-111%). Therefore both formulations were not bioequivalent in all the genotype groups.

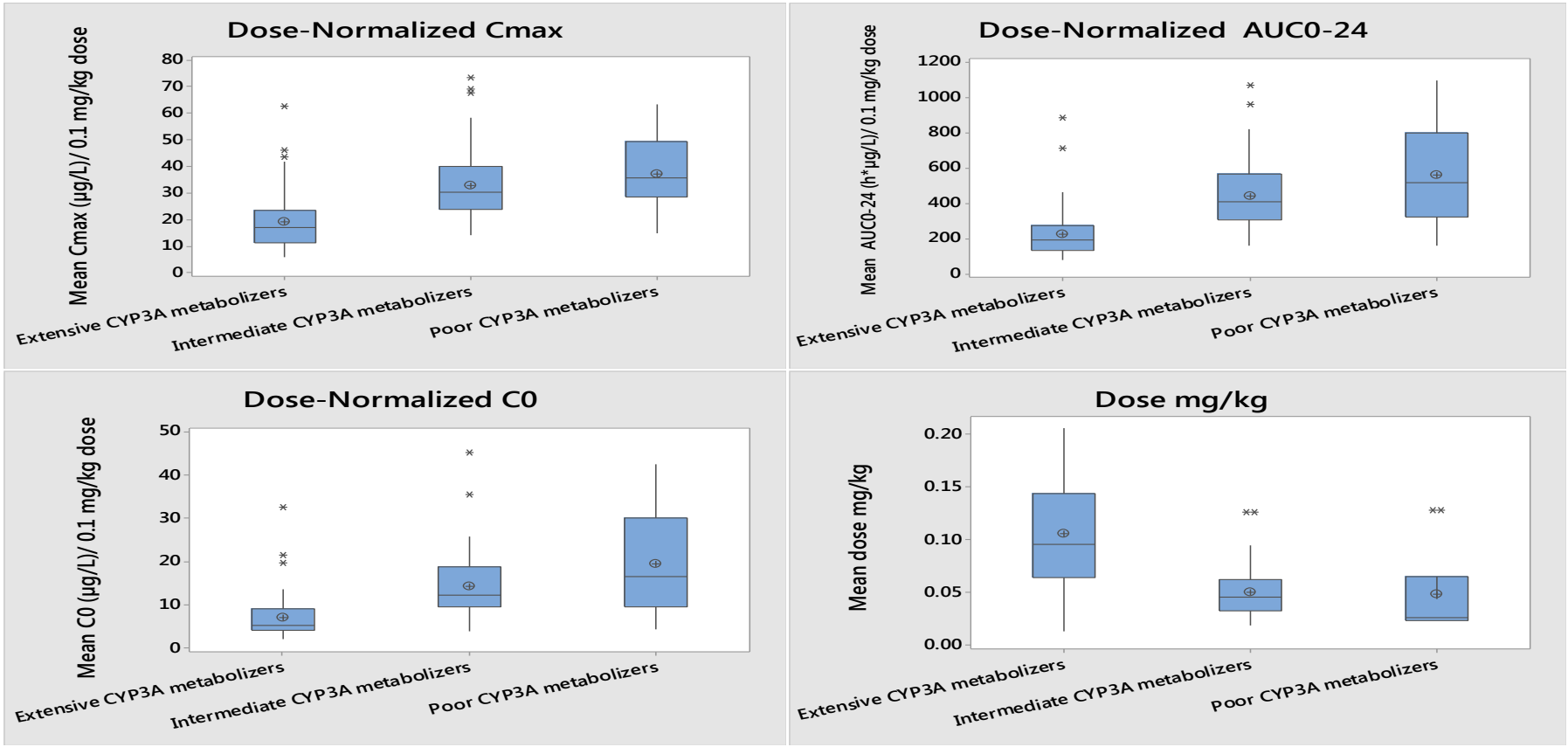


Figure 38: *CYP3A5*3* and *CYP3A4*22* Combined Genotypes and Dose-Normalized Tacrolimus Exposure and Dose Requirement for the Whole Data of Once-and Twice-Daily Tacrolimus. Boxes represent the interquartile range, line in the box represents the median value, symbol in the box represents the mean value, outer lines represent the minimal and maximal value and the values beyond the whiskers (asterisks) are outliers Extensive CYP3A metabolizers are *CYP3A5*1* allele /*CYP3A4*22CC* carriers. Intermediate CYP3A metabolizers are *CYP3A5*3/*3* /*CYP3A4*22CC* carriers. Poor CYP3A metabolizers are *CYP3A5*3/*3* /*CYP3A4*22CT* carriers.

Table 33: *CYP3A5*3* and *CYP3A4*22* Combined Genotypes and Dose-Normalized Tacrolimus Exposure and Dose for the Whole Data of Once-and Twice-Daily Tacrolimus.

PK-parameter	Extensive metabolizers (n= 60)	Intermediate metabolizers (n= 58)	Poor metabolizers (n= 10)	p-value [a]	p-value [b]
Dose (mg/Kg/day)	0.11 ± 0.05	0.05 ± 0.02	0.05 ± 0.04	<0.001	0.2
AUC_{0-24} (µg*h/L/mg/Kg)	223.4 ± 140.3	440.1 ± 190.3	562 ± 313.3	<0.001	0.3
C_{max} (µg/L/mg/Kg)	19.1 ± 10.6	32.8 ± 13.5	37.2 ± 15.4	<0.001	0.4
C_0 (µg/L/mg/Kg)	6.9 ± 5.1	14.3 ± 7.6	19.5 ± 13.0	<0.001	0.4

Extensive CYP3A metabolizers are *CYP3A5*1* allele /*CYP3A4*22CC* carriers. Intermediate CYP3A metabolizers are *CYP3A5*3/*3* /*CYP3A4*22CC* carriers. Poor CYP3A metabolizers are *CYP3A5*3/*3* /*CYP3A4*22CT* carriers. Data are shown as mean ± SD.

[a] p-Values refer to comparisons to extensive metabolizers.

[b] p-Values refer to comparisons between intermediate and poor metabolizers

Values were compared using ANOVA (General linear model).

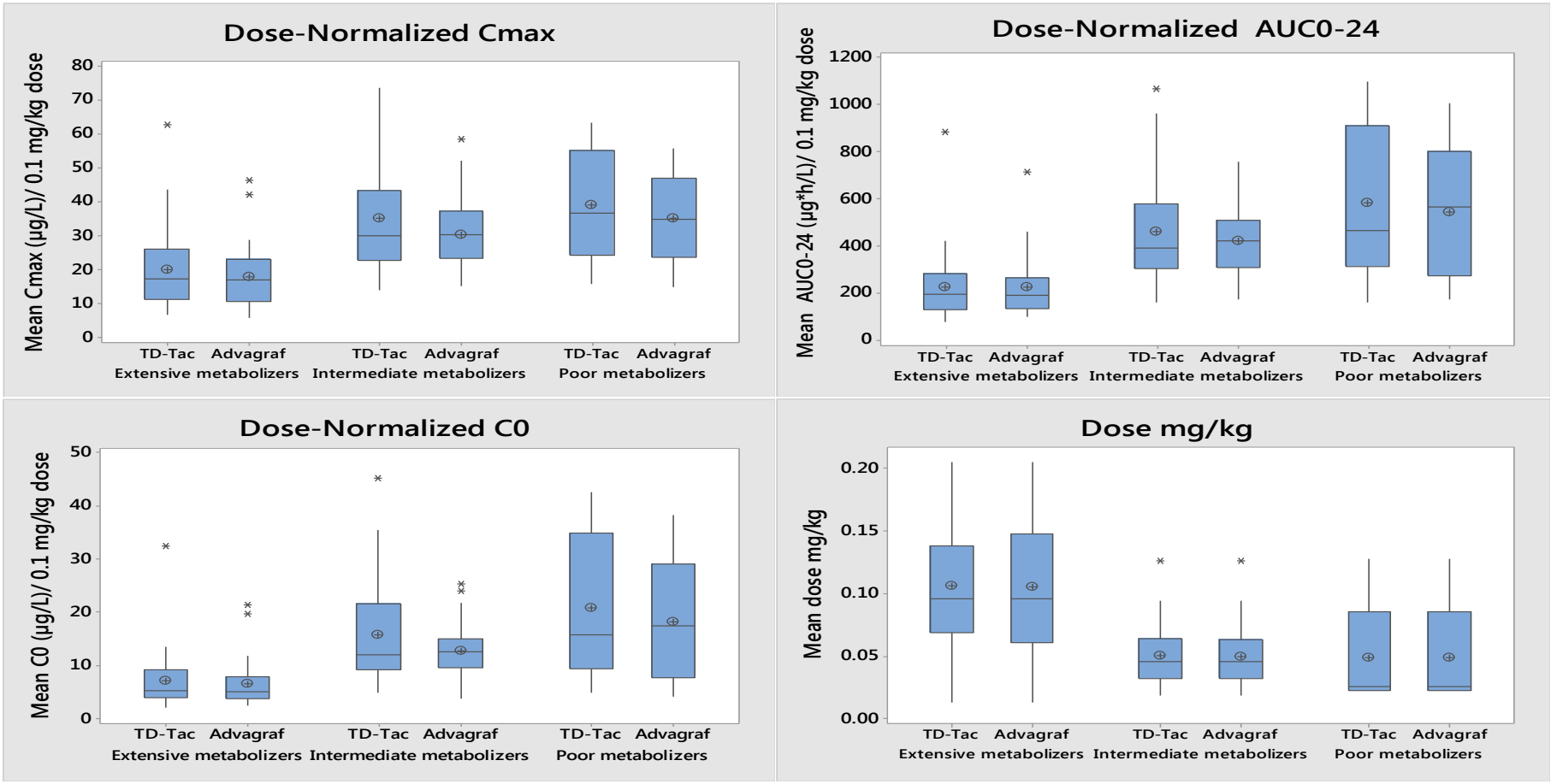


Figure 39: *CYP3A5*3* and *CYP3A4*22* Genotypes Relationship with Dose-Normalized Tacrolimus Exposure and Dose Requirement in Both Tacrolimus Formulations. Boxes represent the interquartile range, line in the box represents the median value, symbol in the box represents the mean value, outer lines represent the minimal and maximal value and the values beyond the whiskers (asterisks) are outliers. Extensive CYP3A metabolizers are *CYP3A5*1* allele /*CYP3A4*22CC* carriers. Intermediate CYP3A metabolizers are *CYP3A5*3/*3* /*CYP3A4*22CC* carriers. Poor CYP3A metabolizers are *CYP3A5*3/*3* /*CYP3A4*22CT* carriers.

Table 34: Tacrolimus Dose-Normalized Pharmacokinetic Parameters for Twice-Daily Tacrolimus (TD-Tac) and Advagraf® in *CYP3A* Combined Genotype Groups

PK-parameter	Extensive metabolizers (n= 30)			Intermediate metabolizers (n= 29)			Poor metabolizers (n= 5)		
	TD-Tac	Advagraf®	p-value	TD-Tac	Advagraf®	p-value	TD-Tac	Advagraf®	p-value
Dose (mg/Kg/day)	0.11 ± 0.05	0.11 ± 0.05		0.05 ± 0.02	0.05 ± 0.02		0.05 ± 0.05	0.05 ± 0.05	
AUC_{0-24} (µg*h/L/mg/Kg)	223 ± 130	224 ± 152	0.9	419 ± 155	461 ± 221	0.6	542 ± 311	582 ± 351	0.9
C_{max} (µg/L/mg/Kg)	18.1 ± 9.2	20.1 ± 11.8	0.6	30.5 ± 10.4	35.2 ± 15.9	0.3	35.3 ± 14.6	39.2 ± 17.6	0.8
C_0 (µg/L/mg/Kg)	6.7 ± 4.5	7.2 ± 5.7	0.7	12.8 ± 5.1	15.8 ± 9.4	0.3	18.2 ± 12.8	20.9 ± 14.5	0.8

Extensive CYP3A metabolizers are *CYP3A5*1* allele /*CYP3A4*22CC* carriers. Intermediate CYP3A metabolizers are *CYP3A5*3/*3* /*CYP3A4*22CC* carriers. Poor CYP3A metabolizers are *CYP3A5*3/*3* /*CYP3A4*22CT* carriers. Data are shown as mean ± SD. Values were compared using ANOVA (General linear model).

Table 35: Bioequivalence Statistics for $AUC_{0–24}$ and C_{max} for Twice-Daily Tacrolimus (TD-Tac) and Once-Daily Tacrolimus OD-Tac in CYP3A Combined Genotype Groups.

Parameter	Extensive metabolizers		Intermediate metabolizers	
	Ratio of geometric means (%)	90% CI	Ratio of geometric means (%)	90% CI
$AUC_{0–24}$	101%	(88% – 116%)	92%	(81% – 104%)
C_{max}	93%	(77% – 111%)	88%	(74% – 104%)

Extensive CYP3A metabolizers are *CYP3A5*1* allele /*CYP3A4*22CC* carriers. Intermediate CYP3A metabolizers are *CYP3A5*3/*3* /*CYP3A4*22CC* carriers.

90% CI for geometric mean are based on the ANOVA model (General linear model).

*5.2.3.4 The Relationship between POR*28 Polymorphism and Tacrolimus Pharmacokinetics*

The analysis has been done on 128 24-h PK profiles obtained from 64 patients with data for TD-Tac and OD-Tac pooled. The relationship between *POR*28* polymorphism and the pharmacokinetic parameters of tacrolimus is shown in **Table 36**. When considering only the *POR*28* allelic status, no significant difference in the dose-normalized AUC_{0-24}, C_{max}, C_0 values and the daily of tacrolimus was observed between the *POR*28* genotype groups ($p > 0.05$). The mean daily dose and plasma concentrations of tacrolimus in individuals with different *POR*28* genotypes are shown in **Figure 40**. When considering both *POR*28* allelic status and tacrolimus formulations, this difference remained not significant (**Table 37** & **Figure 41)**.

In *POR*28 CC* group, AUC_{0-24} OD-Tac / AUC_{0-24} TD-Tac was 97% (90% CI 85% -112%) and C_{max} OD-Tac / C_{max} TD-Tac was 88% (90% CI 75% -103%). While in the *POR*28 CT/TT* group, AUC_{0-24} OD-Tac / AUC_{0-24} TD-Tac was 96% (90% CI 84% -109%) and C_{max} OD-Tac / C_{max} TD-Tac was 93% (90% CI 77% -111%). The C_{max} OD-Tac/ C_{max} TD-Tac ratios in both groups were falling outside 80% to 125% bioequivalence limits. Consequently, both formulations were not bioequivalent. Based on EMA guidelines, the confidence intervals of the AUC_{0-24} ratios were outside the bioequivalence margin (90-111%). Therefore, both formulations were not bioequivalent. These data are summarized in **Table 38.**

Table 36: Dose-Normalized Pharmacokinetics of Tacrolimus in Renal Transplant Recipients with Different *POR*28* Genotypes for the Whole Data of Once-and Twice-Daily Tacrolimus.

PK-parameter	*POR*28 CC* (n= 60)	*POR*28 CT/TT* (n= 68)	p-value
Dose (mg/Kg/day)	0.08 ± 0.05	0.07 ± 0.05	0.72
C_{max} (µg/L/mg/Kg)	26.6 ± 14.5	26.9 ± 14.2	0.96
AUC_{0-24} (µg*h/L/mg/Kg)	353 ± 239	344 ± 198	0.68
C_0 (µg/L/mg/Kg)	11.8 ± 9.6	10.8 ± 7.0	0.89

Data are shown as mean ± SD. Values were compared using ANOVA (General linear model).

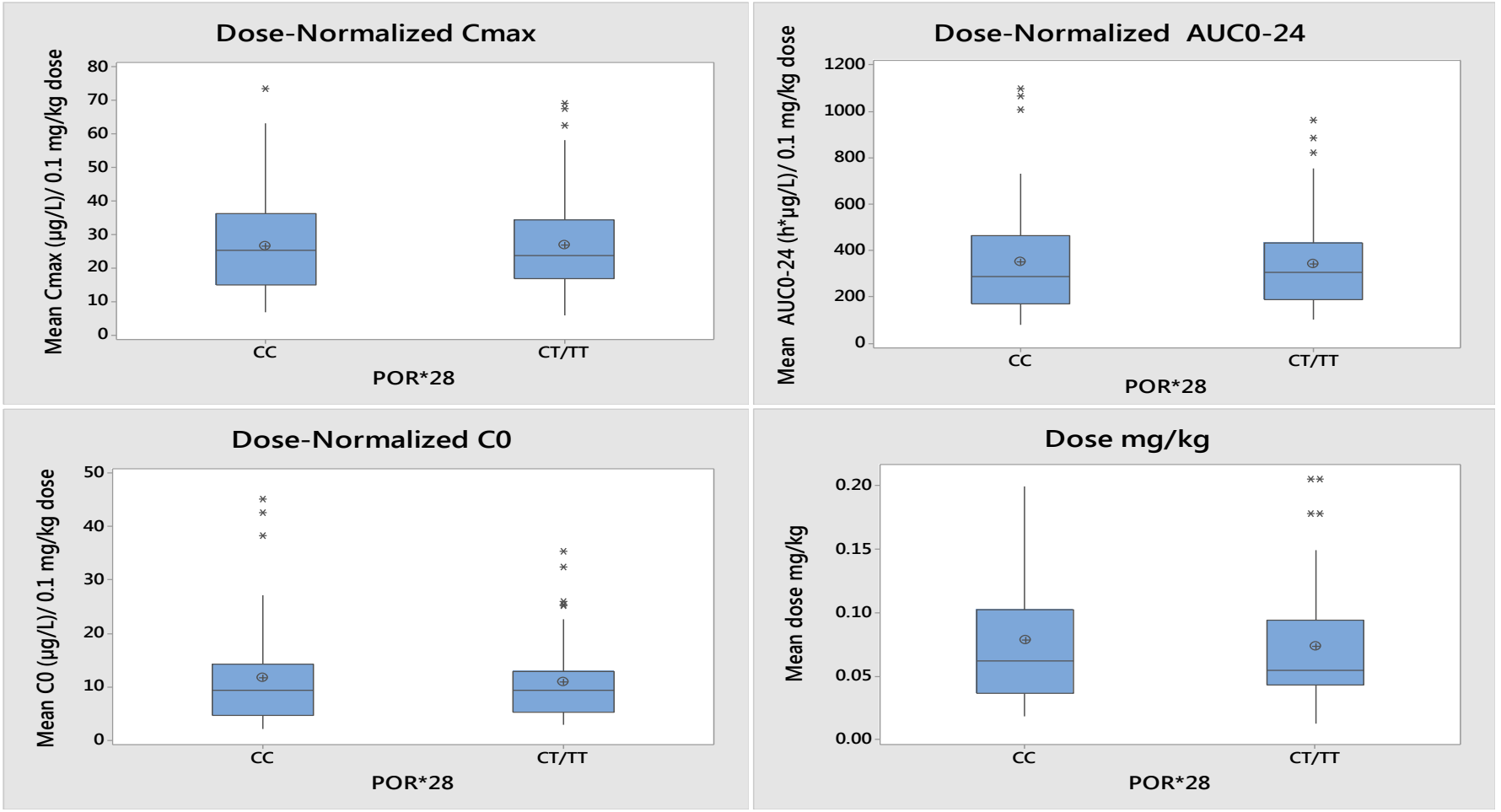


Figure 40: The Dose-Normalized Mean Pharmacokinetic Parameters of Tacrolimus in Renal Transplant Recipients with Different *POR*28* Genotypes for the Whole Data of Once-and Twice-Daily Tacrolimus. Boxes represent the interquartile range, line in the box represents the median value, symbol in the box represents the mean value, outer lines represent the minimal and maximal value and the values beyond the whiskers (asterisks) are outliers.

Table 37: Correlation of *POR*28* Polymorphism and Tacrolimus Formulation with Dose-Normalized Tacrolimus Pharmacokinetic Parameters.

PK-parameter	*POR*28 CC* (n= 30)			*POR*28 CT/TT* (n= 34)		
	TD-Tac	**Advagraf®**	**p-value**	**TD-Tac**	**Advagraf®**	**p-value**
Dose (mg/Kg/day)	0.08 ± 0.05	0.08 ± 0.05		0.07 ± 0.05	0.07 ± 0.05	
$AUC_{0\text{-}24}$ (µg*h/L/mg/Kg)	361 ± 260	344 ± 221	0.94	357 ± 226	331 ± 168	0.81
C_{max} (µg/L/mg/Kg)	28.7 ± 16.4	24.5 ± 12.3	0.44	28.2 ± 16.2	25.5 ± 12.0	0.59
C_0 (µg/L/mg/Kg)	12.5 ± 10.9	11.0 ± 8.1	0.76	11.9 ± 8.4	9.8 ± 5.3	0.40

TD-Tac: Twice daily tacrolimus.
Values were compared using ANOVA (General linear model).

Table 38: Bioequivalence Statistics for $AUC_{0–24}$ and C_{max} for TD-Tac and OD-Tac in *POR*28* Genotypes.

Parameter	*POR*28 CC*		*POR*28 CT/TT*	
	Ratio of geometric means (%)	**90% CI**	**Ratio of geometric means (%)**	**90% CI**
$AUC_{0–24}$	97%	(85% – 112%)	96%	(84% – 109%)
C_{max}	88%	(75% – 103%)	93%	(77% – 111%)

90% CI for geometric mean are based on the ANOVA model (General linear model).

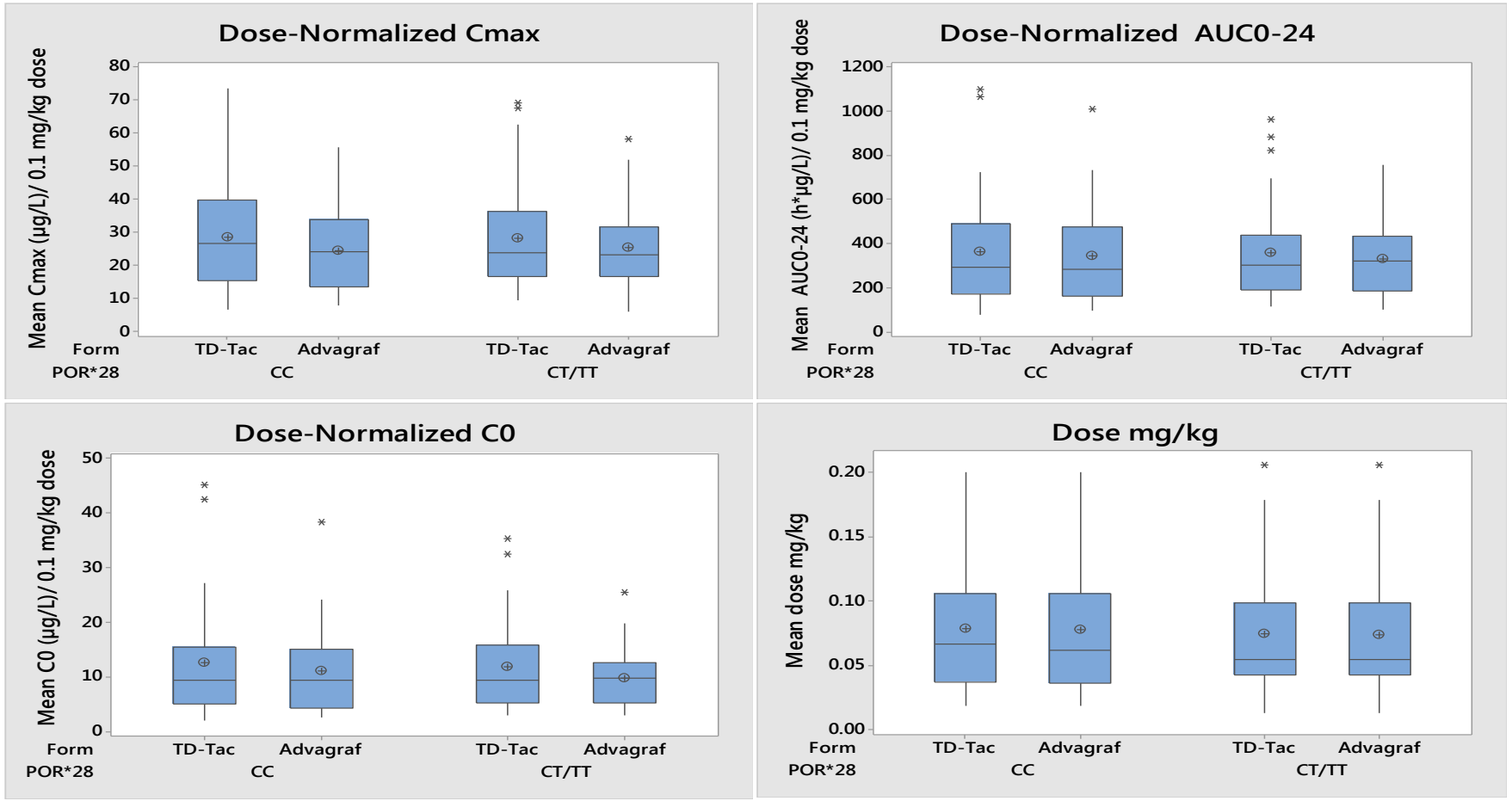


Figure 41: Dose-Normalized Mean Pharmacokinetic Parameters of Tacrolimus with Different *POR*28* Genotypes in Once- and Twice-Daily Tacrolimus. Boxes represent the interquartile range, line in the box represents the median value, symbol in the box represents the mean value, outer lines represent the minimal and maximal value and the values beyond the whiskers (asterisks) are outliers.

*5.2.3.5 POR*28 Polymorphism Association with Tacrolimus Pharmacokinetics in CYP3A5 Expressers and Non-Expressers.*

According to the likely level of CYP3A5 expression, subjects were divided into CYP3A5 expressers (*CYP3A5*1* allele carriers) and CYP3A5 non-expressers (*CYP3A5*3/*3* carriers). The effect of the *POR*28* polymorphism on the pharmacokinetics of tacrolimus was studied in each group. The demographic characteristics of each subgroup are shown in **Table 39**. We observed no differences in these characteristics between *POR*28* genotypes in both CYP3A5 expressers and non-expressers. The analysis has been done on 128 24-h PK profiles obtained from 64 patients with data for TD-Tac and OD-Tac pooled.

CYP3A5 non-expressers carrying at least one *POR*28 T* allele had a significant increase in tacrolimus daily dose compared to CYP3A5 non-expressers carrying the *POR*28CC* genotype [median (IQR): 0.05 (0.02 – 0.13) mg/Kg/day vs. 0.04 (0.02 – 0.08) mg/Kg/day; $P < 0.01$). In CYP3A5 non-expressers, the dose-normalized tacrolimus C_0 was significantly higher in *POR*28 CC* carriers than *POR*28 T* variant allele carriers (18.7 ± 10.3 vs. 12.9 ± 6.7, $P<0.01$). There was no statistically significant association between *POR*28* genotype and the CYP3A5 expresser group. The dose-normalized tacrolimus AUC_{0-24} for *POR*28 T* allele carriers expressing CYP3A5 was not significantly different to that in *POR*28 CC* homozygote patients ($P = 0.41$). By contrast to the CYP3A5 expresser group, *POR*28 CT/TT* individuals not expressing CYP3A5 had a 24% reduction in the dose-normalized AUC_{0-24} (90%CI 4 to 40%; $p<0.01$). Moreover, no significant difference was found in tacrolimus dose-normalized C_{max} between the *POR*28* genotype groups, neither in the CYP3A5 expresser group ($P = 0.72$) nor in the CYP3A5 non-expresser group ($P = 0.16$); See **Figure 42** & **Table 40.**

Table 39: Patients Demographic Characteristics According to *CYP3A5* and *POR*28* Genotypes.

Characteristic	**CYP3A5 Expressers (**1/*1* & **1/*3*)**		**CYP3A5 Non-expressers (**3/*3*)**	
	***POR*28 CC* (n=17)**	***POR*28 CT/TT* (n=13)**	***POR*28 CC* (n=13)**	***POR*28 CT/TT* (n= 21)**
Sex				
Male/female	10/7	8/5	9/4	16/5
Age (y) , mean (SD)	51.7 ± 11.1	54.8 ± 14.2	57.2 ± 15.5	55.0 ± 11.5
Race, n (%)				
White	10 (59%)	9 (69%)	7 (54%)	13 (62%)
Black	4 (24%)	2 (15%)	3 (23%)	3 (14 %)
Asian	3 (18%)	2 (15%)	3 (23%)	5 (24 %)
Body weight (kg), mean (SD)	83.4 ± 19.5	73.6±9.4	72.1 ± 9.1	75.1 ± 16.1
Height (cm), mean (SD)	171.7 ± 9.5	168.4±8.6	173.0 ± 9.9	168.2 ± 6.5
Diabetes, n (%)	3 (18%)	3 (23%)	3 (23%)	6 (29%)
Time since transplantation (years)				
Mean (SD)	3.6 ± 5.7	6.2 ± 4.2	3.8 ± 4.0	3.4 ± 4.2
Median (interquartile range, QR)	1.3	6.1	1.1	1.7
Donor type, n				
Living / Deceased	10/7	4/9	5/8	10/11
Serum creatinine (µmol/L)	125.1 ± 33.4	112 ± 27	122.2 ± 61.9	132.9 ± 33.2
Serum albumin(g/L)	38.7 ± 2.6	38.4 ± 3.2	38.3 ± 2.4	39.4 ± 3.8
Haemoglobin, (g/L)	129.3 ± 14.1	140.2 ± 18.1	127.5 ± 13.5	124.5 ± 31.6
Immunosuppression therapy				
Tacrolimus, n (%)				
Prograf®/ Adoport®	10 (59%) / 7 (41%)	11 (85%) / 2 (15%)	10 (77%) / 3 (23%)	16 (76%) / 5 (24%)
Corticosteroids, n (%)	5 (29%)	13 (100%)	10 (77%)	11 (52%)
Azathioprine, n (%)	6 (35%)	_	3 (23%)	5 (24%)
Mycophenolate mofetil, n (%)	7 (41%)	2 (6%)	4 (31%)	6 (29%)
***CYP3A4*22* Polymorphism**				
*CYP3A4*22 CC*	17 (100%)	13 (100%)	10 (77%)	19 (90%)
*CYP3A4*22 CT*	0 (0%)	0 (0%)	3 (23%)	2 (10%)

Table 40: Dose-Normalized Tacrolimus PK Parameters According to *CYP3A5*3* and *POR*28* Genotypes for the Whole Data of Once-and Twice-Daily Tacrolimus.

PK-parameter	CYP3A5 Expressers (**1/*1 & *1/*3*)		p-value	CYP3A5 Non-expressers (**3/*3*)		p-value
	POR*28 CC **(n= 34)**	***POR*28 CT/TT*** **(n= 26)**		***POR*28 CC*** **(n= 26)**	***POR*28 CT/TT*** **(n= 42)**	
Dose (mg/Kg/day)	0.11 ± 0.05	0.10 ± 0.05	0.56	0.04 ± 0.02	0.06 ± 0.03	0.006
C_{max} (µg/L/mg/Kg)	19.1 ± 10.2	19.1 ± 11.2	0.72	36.4 ± 13.6	31.7 ± 13.8	0.16
AUC_{0-24} (µg*h/L/mg/Kg)	212 ± 105.7	238 ± 177	0.41	537 ± 241	409 ± 183	0.02
C_0 (µg/L/mg/Kg)	6.5 ± 4.0	7.5 ± 6.3	0.40	18.7 ± 10.3	12.9 ± 6.7	0.007

Values were compared using ANOVA (General linear model).

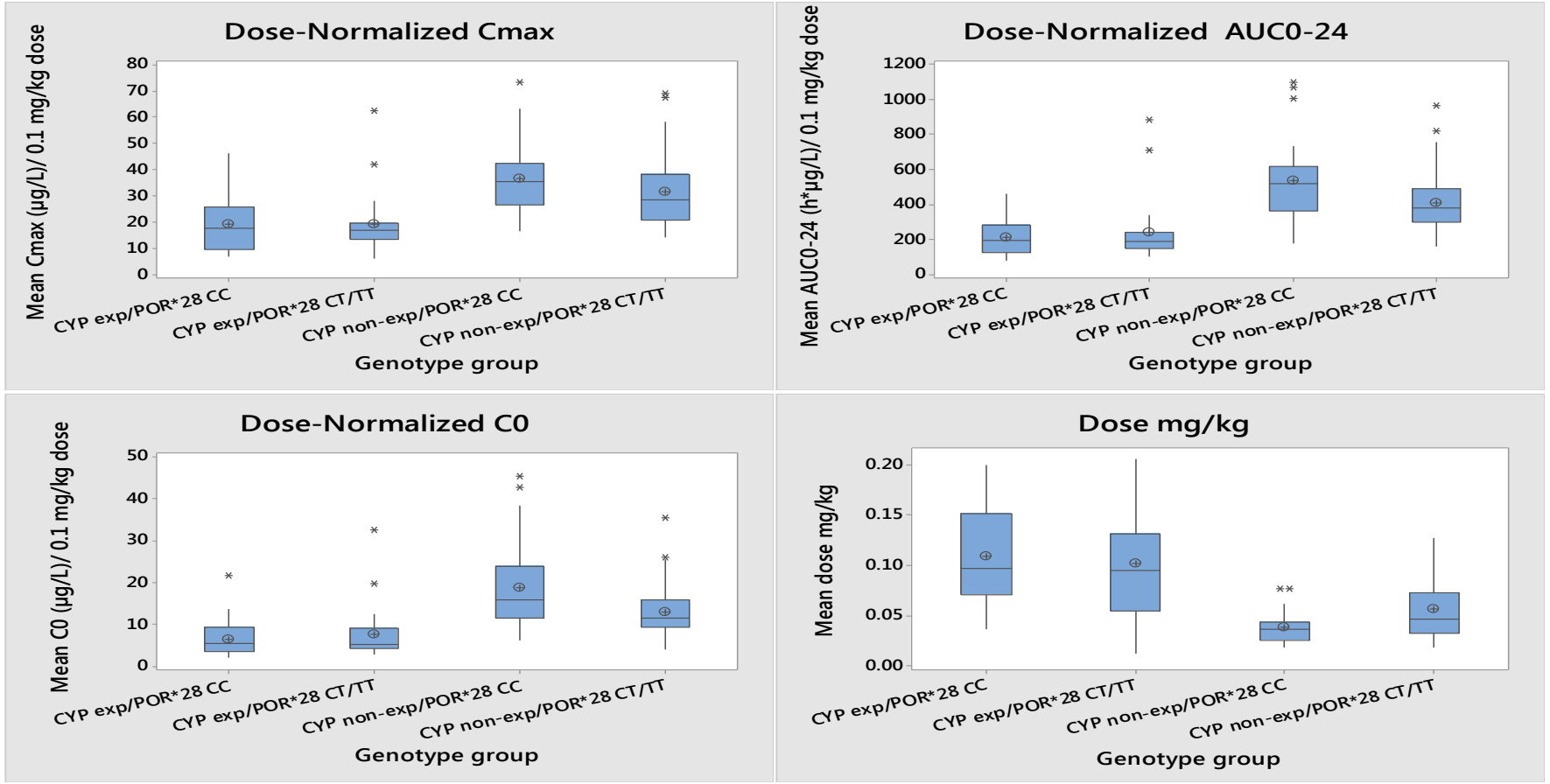


Figure 42: Dose-Normalized Mean Pharmacokinetic Parameters of Tacrolimus in Different *CYP3A5* and *POR*28* Combined Genotypes for the Whole Data of Once-and Twice-Daily Tacrolimus. Boxes represent the interquartile range, line in the box represents the median value, symbol in the box represents the mean value, outer lines represent the minimal and maximal value and the values beyond the whiskers (asterisks) are outliers. CYP exp refers to CYP3A5 expressers (*CYP3A5 *1/*1* & *1/*3* carriers) and CYP non-exp refers to CYP3A5 non-expressers (*CYP3A5*3/*3* carriers).

Additionally, when considering both the combined effects of *CYP3A5*3* and *POR*28* allelic status and tacrolimus formulations, no significant difference in tacrolimus dose-normalized pharmacokinetic parameters and dose was found between the *POR*28* genotypes within both the CYP3A5 expressers and non-expressers (P >0.05; **Table 41 & Figure 43**).

In CYP3A5 expressers carrying *POR*28 CC* genotype, the ratios of geometric means were 104% (90% CI of 88 –123%, p = 0.77) for $AUC_{0–24}$ and 92% (90% CI 74 –115%, p = 0.71) for C_{max}. For *POR*28 CT/TT* carriers the ratios of geometric means were 98% (90% CI of 71 –136%, p = 0.92) for $AUC_{0–24}$ and 93% (90% CI 59 –146%, p = 0.70) for C_{max}. While in CYP3A5 non-expressers carrying *POR*28 CC* genotype, the ratios of geometric means were 98% (90% CI of 71% –136%, p = 0.21 for $AUC_{0–24}$ and 82% (90% CI 64–106%, p = 0. 57) for C_{max}. For *POR*28 CT/TT* carriers the ratios of geometric means were 94% (90% CI of 80–112%, p = 0.64) for $AUC_{0–24}$ and 92% (90% CI 73 –117%%, p = 0.78) for C_{max}. The confidence intervals of AUC_{0-24} OD-Tac / AUC_{0-24} TD-Tac ratios were within the bioequivalence limits in CYP3A5 expressers having *POR*28 CC* genotype and CYP3A5 non-expressers having *POR*28 CT/TT* genotype. Whereas the confidence intervals of the other ratios were falling outside 80% to 125% bioequivalence limits. As a result, both formulations were not bioequivalent in all the genotype groups. Based on EMA guidelines, the confidence intervals of the AUC_{0-24} ratios were outside the bioequivalence margin (90-111%) in all the genotype groups. Consequently, the two formulations were not bioequivalent. These data are summarized in **Table 42**.

Table 41: Dose-Normalized Tacrolimus Pharmacokinetic Parameters for Different Combination of *CYP3A5*3* and *POR*28* Genotypes in Both Once- and Twice-Daily Tacrolimus.

PK-parameter	CYP3A5 Expressers (*1/*1 & *1/*3)					
	*POR*28 CC* (n=17)			*POR*28 CT/TT* (n=13)		
	TD-Tac	**Advagraf®**	**P-value**	**TD-Tac**	**Advagraf®**	**P-value**
Dose (mg/Kg/day)	0.11 ± 0.05	0.11 ± 0.05		0.10 ± 0.05	0.10 ± 0.05	
C_0 (µg/L/mg/Kg)	6.4 ± 3.4	6.6 ± 4.7	0.96	8.2 ± 7.8	6.8 ± 4.5	0. 66
C_{max} (µg/L/mg/Kg)	19.9 ± 10.5	18.3 ± 10.0	0. 71	20.2 ± 13.8	17.9 ± 8.3	0.70
AUC_{0-24} (µg*h/L/mg/Kg)	207 ± 105	216 ± 110	0. 77	245 ± 201	232 ± 158	0.92
PK-parameter	**CYP3A5 Non-expressers (*3/*3)**					
	*POR*28 CC* (n=13)			*POR*28 CT/TT* (n=21)		
	TD-Tac	**Advagraf®**	**P-value**	**TD-Tac**	**Advagraf®**	**P-value**
Dose (mg/Kg/day)	0.04 ± 0.02	0.04 ± 0.02		0.06 ± 0.03	0.06 ± 0.03	
C_0 (µg/L/mg/Kg)	20.5 ± 12.2	16.8 ± 8.1	0.51	14.1 ± 8.1	11.6 ± 4.9	0.38
C_{max} (µg/L/mg/Kg)	40.1 ± 15.8	32.7 ± 10.3	0.21	33.1 ± 15.8	30.2 ± 11.6	0.64
AUC_{0-24} (µg*h/L/mg/Kg)	563 ± 266	511 ± 220	0.57	427 ± 216	392 ± 146	0.78

TD-Tac: Twice daily tacrolimus.
Values were compared using ANOVA (General linear model).

Table 42: Ratios of Geometric Means and 90% CI for AUC_{0-24} and C_{max} for Tacrolimus Formulations in *CYP3A5* and *POR*28* Genotype Groups

Parameter	CYP3A5 Expressers (**1/*1* & **1/*3*)			
	POR*28 CC		***POR*28 CT/TT***	
	Ratio of geometric means (%)	**90% CI**	**Ratio of geometric means (%)**	**90% CI**
AUC_{0-24}	104%	(88% – 123%)	98%	(71% – 136%)
C_{max}	92%	(74% – 115%)	93%	(59% – 146%)
Parameter	**CYP3A5 Non-expressers (**3/*3*)**			
	POR*28 CC		***POR*28 CT/TT***	
	Ratio of geometric means (%)	**90% CI**	**Ratio of geometric means (%)**	**90% CI**
AUC_{0-24}	98%	(71% – 136%)	94%	(80% – 112%)
C_{max}	82%	(64% – 106%)	92%	(73% – 117%)

90% CI for geometric mean are based on the ANOVA model (General linear model).

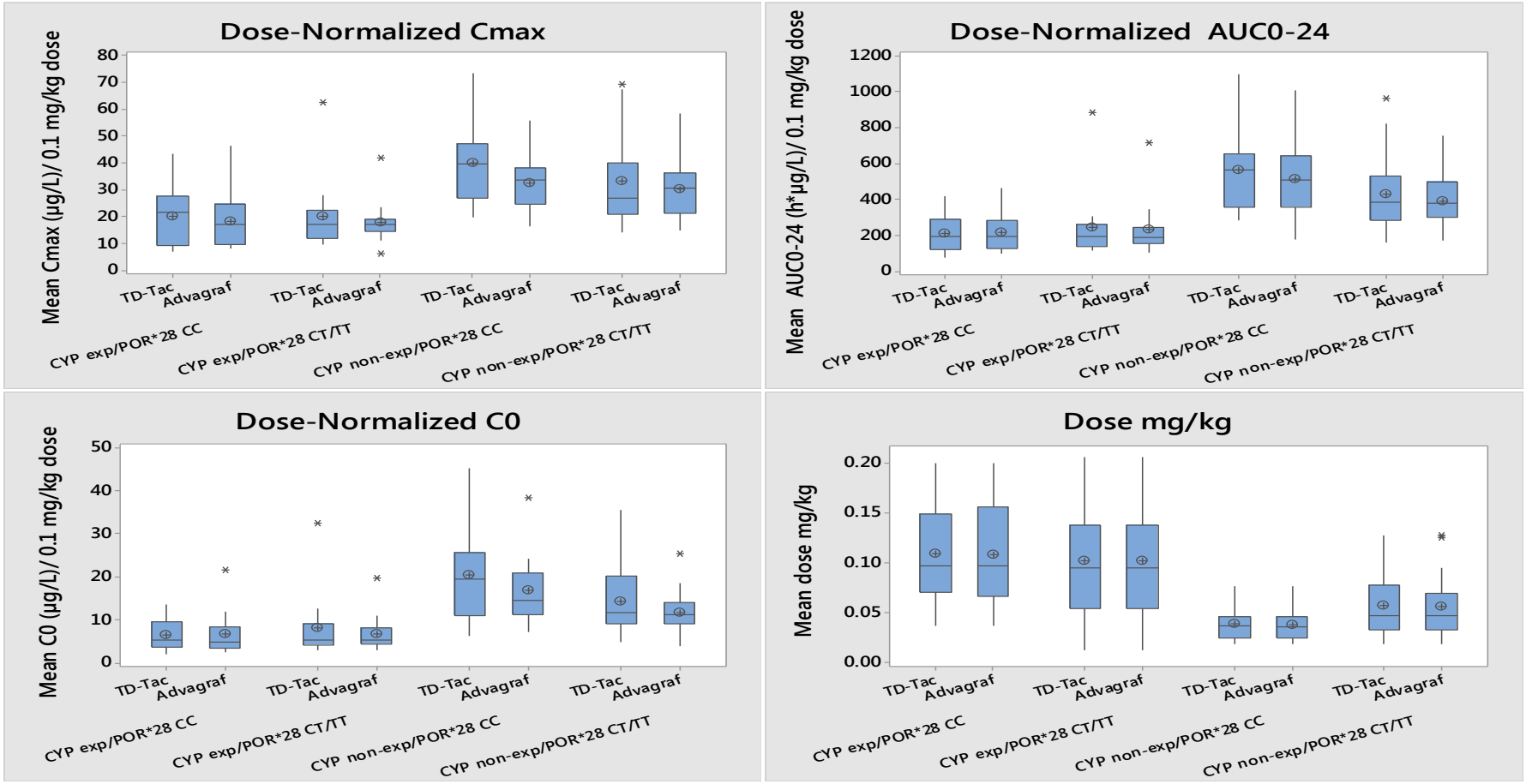


Figure 43: Dose-Normalized Mean Pharmacokinetic Parameters of Tacrolimus with Different *CYP3A5*3* and *POR*28* Combined Genotypes in Twice-Daily Tacrolimus (TD-Tac) and Advagrf®. Boxes represent the interquartile range, line in the box represents the median value, symbol in the box represents the mean value, and outer lines represent the minimal and maximal value and the values beyond the whiskers (asterisks) are outliers. CYP exp refers to CYP3A5 expressers (*CYP3A5 *1/*1* & *1/*3* carriers) and CYP non-exp refers to CYP3A5 non-expressers (*CYP3A5*3/*3* carriers).

5.2.3.6 Factors Associated with Dose Requirements of Tacrolimus.

The analysis has been done on 128 24-h PK profiles obtained from 64 patients with data for TD-Tac and OD-Tac pooled. As we mentioned before, univariate regression analysis showed that age, sex, haematocrit, ethnicity, time since transplant, *ABCB1 3435* and *CYP3A5*3* are factors associated with tacrolimus dose requirements. However, *CYP3A4*22* served as a borderline significant factor (p= 0.06) and *POR*28* had no association with tacrolimus dose requirement.

In multiple regression analysis by stepwise selection, neither *CYP3A4*22* nor *POR*28* had any association with tacrolimus dose requirement. Whereas multiple regression analysis identified the combined *CYP3A5*3*/ *ABCB1 3435* genotypes, age, sex, ethnicity, haematocrit, corticosteroids treatment and diabetic status as independent variables associated with tacrolimus dose (**Table 27**) as mentioned before in **chapter 4**. Therefore, these independent factors explain exactly the same percentage of variability in tacrolimus dose requirements (59.9%) as shown in **chapter 4**.

5.2.4 Discussion

In addition to CYP3A5 activity which explains 29–35% of the variability in the studied tacrolimus pharmacokinetic parameter. CYP3A4 activity, explains an additional 23–29% of the variability in the studied tacrolimus pharmacokinetic parameters (de Jonge et al., 2012). In line with recent studies that demonstrated increased tacrolimus daily dose and dose-adjusted concentrations in renal transplant recipients carrying a *CYP3A4*22* T variant allele (Elens et al., 2011b) , we found that there was a significant reduction in tacrolimus adjusted dose in *CYP3A4*22 CT* than *CC* genotype. Similarly, we found that tacrolimus dose-normalized C_0, C_{max} and AUC_{0-24} were significantly lower in *CYP3A4*22 CC* in comparison with *CYP3A4*22 CT* variant. Those results were also confirmed by Elens colleague in another study in kidney transplant patients. They found that *CYP3A4*22* polymorphism significantly altered tacrolimus metabolism and dose requirement early after transplantation, T allele carriers require 33% lower tacrolimus doses than wild-type CC carriers to reach the target C_0 (Elens et al., 2011a). Furthermore, de Jonge et al. (2014) reported that steady-state oral clearance of

tacrolimus was reduced in *CYP3A4*22* T-allele carriers compared with CC carriers, resulting in 50% lower tacrolimus daily dose requirements. However, different results were reported by Santoro et al. (2013) who did not find any association between *CYP3A4*22* and tacrolimus C_0/dose, Beatriz et al. (2013) who found no significant differences between the *CYP3A4*22* genotypes and daily dose requirement and dose-normalized C_0 and Tavira et al. (2013) who reported no association between *CYP3A4*22* variant and tacrolimus dose at 1week and 6 month after transplantation. It is not clear why these authors found no association, but sample size may have played a part.

CYP3A4 mRNA level and enzyme activity in livers with CC genotype were reported as 1.7- and 2.5-fold, respectively, greater than in CT and TT carriers (Wang et al., 2011). CYP3A4 variants that result in higher enzymatic activities could be linked to reduced tacrolimus blood concentration. The discrepancy between our results and other studies, might be due to the absence of *CYP3A4*22* TT variants or could be attributed to the very high *CYP3A4*22 CC* and low *CYP3A4*22 CT* allelic frequencies in our subjects population. However, statistical power is not likely to be a key limiting factor in the current study. A confounding factor is the fact that none of the CT heterozygotes were CYP3A5 expressers. When combining *CYP3A5* and *CYP3A4*22*, we found a significantly higher daily dose of tacrolimus in extensive CYP3A metabolizers compared to the intermediate CYP3A metabolizers and poor CYP3A metabolizers. After excluding CYP3A5 expressers having *CYP3A4*22 CC* carriers (Extensive CYP3A metabolizers) from the analysis we found no significant differences in tacrolimus dose and pharmacokinetics between the *CYP3A4*22 CC* (intermediate CYP3A metabolizers) and the *CT* (poor CYP3A metabolizers) genotype subgroups in CYP3A5 non-expresser group, suggesting that the significant difference in tacrolimus pharmacokinetics between *CYP3A4*22 CC* and *CYP3A4*22 CT* was due to the fact that all *CYP3A4*22T* allele carriers are CYP3A5 non-expressers. Similar findings were obtained by Gijsen et al. (2013) showing no significant difference between poor and intermediate metabolites. In contrast to our findings, Elens et al. (2011b) reported a strong correlation between CYP3A combined genotypes and tacrolimus disposition. CYP3A poor metabolizer group presented dose-adjusted tacrolimus concentration 1.6-

and 4.1-fold higher than the intermediate metabolizer and extensive metabolizer groups, respectively. This was confirmed by another study showing that CYP3A extensive metabolizers required 2.1-fold higher doses than intermediate CYP3A metabolizers or 2.7-fold higher doses than CYP3A poor metabolizers (Bruckmueller et al., 2014).

It is well-known that P450 oxidoreductase (POR) is essential for cytochrome P450 (CYP) activity in humans and it has been associated with increased in vivo CYP3A activity (de Jonge et al., 2011). In this work, we studied the effect of the *POR*28* genetic polymorphism on the pharmacokinetics of tacrolimus. Up to date, very few studies were carried out to explore the influence of genetic variations in the POR gene on tacrolimus metabolism. Our findings showed that there were no significant differences in the pharmacokinetics between the *POR*28 CC* genotype and the *POR*28 CT/TT* genotype with respect to dose-adjusted C_{max}, AUC_{0-24}, tacrolimus trough blood concentrations and the daily dose (mg/kg). This confirms the previous results obtained after a single dose tacrolimus in a cohort of healthy Chinese volunteers (Zhang et al., 2013). Conversely, another study demonstrated that the *POR*28* SNP was associated with significant increases in early tacrolimus dose-requirements in patients carrying a *CYP3A5*1* allele (CYP3A5 expressers) and had no effect on tacrolimus trough blood concentrations and daily dose requirements in CYP3A5 non-expressers (de Jonge et al., 2011). However, our findings were in line with previous observations by Elens et al. (2014) who found that in the CYP3A5 expresser group, *POR*28* carriers (*POR*1/*28* and *POR*28/*28)* had low tacrolimus dose-adjusted C_0 when compared with *POR*1/*1* patients. They also found a significant decrease in dose-adjusted C_0 in *POR*28/*28* individuals not expressing CYP3A5 compared to *POR*1/*28* and *POR*1/*1*carriers. The inconsistency between these results and our findings might be due to our relatively small study population. The inconsistency in the *POR*28* influence makes it a poor candidate for inclusion in pharmacogenetic algorithms based on currently available data. More powerful studies are needed to reach a definitive conclusion.

Chapter 6. 4β-hydroxycholesterol Measurement as a Potential Biomarker for CYP3A4 and CYP3A5 Activity in Informing Tacrolimus Dosing.

6.1 Introduction

CYP3A enzymes exhibit a large variation in hepatic expression and biological activity between different individuals (Diczfalusy et al., 2009). 4β-hydroxycholesterol (4β-OHC) concentration increases with the number of active *CYP3A5*1* alleles. Recently, 4β-HC has been shown to be an endogenous marker of P450 3A activity in clinical practice and it is appropriate for the assessment of CYP3A activity in stable kidney transplant recipients (Diczfalusy et al., 2011).

6.2 Objective of the Study:

The aim of this study was to investigate the relationship between genetically determined variation in CYP3A expression in comparison to the phenotypic marker 4β-OHC and tacrolimus pharmacokinetics and dose requirement in adult renal transplantation recipients.

6.3 Materials and Methods

6.3.1 Patients and Study Design

This study is part of a larger pharmacokinetic study; detailed information on the study participants and procedure can be found in **Chapter 4.** None of the patients were treated with any medications known to inhibit or induce CYP3A activity. All the study samples were collected at 12.5 hour post-dose for twice daily tacrolimus. Five mL of EDTA anticoagulated blood was collected for 4β-hydroxycholesterol measurement. Plasma samples were prepared by centrifugation for 10 minutes at 2500 g at room temperature and stored frozen at approximately -20ºC until analysis.

6.3.2 4β-hydroxycholesterol Analysis

This study has been conducted in collaboration with Professor Ron van Schaik's team, in Rotterdam. As we had some difficulty in establishing this new method in our laboratory, I had to travel to Professor Ron van Schaik's laboratory at Erasmus MC University of Netherlands in Rotterdam asking for the assistance from his team who validated the method. I have been trained by Evert de Jonge on this analytical method and he supervised me while doing the analysis of my samples.

This analysis procedure was conducted following the method previously validated at Clinical Chemistry Department; Erasmus MC University of Netherlands titled "Quantification of endogenous CYP3A marker 4β-hydroxycholesterol in human plasma by LC-ESI-MS/MS using picolinyl derivatisation." It allows the analysis of the plasma 4β-hydroxycholesterol at concentrations ranging from 4.3-137 µg/L with a correlation coefficient of 0.999. The lower limit of quantitation was 1.8 µg/L. Both the within-day and between-day precision values were all < 15%.

6.3.2.1 Instrumentation

Solvent delivery was achieved using a Waters Acquity™ binary solvent manager set at 0.3mL/minute. Sample injection was performed using Waters Acquity™ sample organizer and sample manager which adjusted to keep samples at 15ºC. Chromatography was on an Acquity ultra-performance liquid chromatography (UPLC®) BEH phenyl, 1.7µm, 2.1×100 mm column (Waters, Etten-Leur, The Netherlands) maintained at 27ºC with Waters Acquity™ column manager. Detection was by a Waters Quattro Premier XE™ Mass spectrometer (Waters, Etten-Leur, The Netherlands). MassLynx 4.1 software was used to control the UPLC/MS, record the output from the detector and for the data acquisition, processing and quantification. The MassLynx software was supplied by Waters, The Netherlands. The calibration curves were generated using 4β- OHC to 4β- OHC-d7 peak area ratio versus the nominal 4β-OHC concentrations with linear regression weighting 1/x. MS Settings: A Waters Quattro Premier XE tandem quadrupole mass spectrometer equipped with Z spray interface was

used to introduce the sample into the mass spectrometer. Argon was used as the collision gas.

6.3.2.2 Chemicals and Reagents

4β-hydroxycholesterol was supplied by Steraloids Inc., Newport, USA and 4β-hydroxycholesterol-d7 (I.S) was obtained from Toronto Research Chemical (North York, Canada), while 4α-hydroxycholesterol was synthesized by SYNCOM, Groningen, The Netherlands. LC-MS-grade methanol, acetonitrile and water were obtained from Biosolve BV (Valkenswaard, The Netherlands). Ethanol and n-hexane were supplied by Sigma-Aldrich (Darmstadt, Germany). Sodium hydroxide, formic acid, picolinic acid, pyridine, triethylamine, 2-methyl-6-nitrobenzoic anhydride and 4-dimethylaminopyridine were purchased from sigma-aldrich (Zwijndrecht, The Netherlands). Deionised water was prepared on site (Millipore).

6.3.2.3 Stock Solutions

Calibrators and control samples were prepared in EDTA-anticoagulated plasma, using separate stock solutions prepared in ethanol. Six non-zero calibrators (nominal values of 4.3, 8.6, 17.2, 34.5, 68.9, and 137.8 µg/L) and two control samples (nominal values of 14.5, and 50.4 µg/L) were prepared. Calibrators and controls were aliquoted and stored at approximately -80°C until the day of analysis. Stock solution of 4β-hydroxycholesterol-d7 (internal standard) was prepared in ethanol to give a concentration of 20 mg/mL and then stored at -20°C until use. On the day of the analysis, 250µL of the stock solution was freshly diluted with 50 ml of deionized water to give a concentration of 100 µg/L.

6.3.2.4 Extraction Procedure

For sample preparation, 50µL of calibrators, quality control, or patient samples; 50µL of internal standard and 500µL of 1 M ethanolic potassium hydroxide (5.61 g KOH/100 mL ethanol) were pipetted into a 4.5-mL glass tube. After mixing and incubation at 37°C for 30 minutes, 300µL of deionized water was added and samples were extracted

twice by the addition of 1 mL n-hexane to each tube. In each extraction step the tubes were vigorously shaken for 15 minutes using Denley Reciprocal Mixer (Denley Instruments Ltd) and centrifuged at 800g for 3 minutes. The organic layers were then transferred to 4.5-mL glass tubes, placed in a Savant SpeedVac Plus Model Sc210A (Thermo Life Sciences) at 60°C, and evaporated to dryness. 170 µL of freshly prepared derivatization mixture (10mg 2-methyl-6-nitrobenzoic anhydride, 3mg 4-dimethylaminopyridine, 8mg picolinic acid, 150µL pyridine and 20 µL triethylamine per reaction) was added to the dried residues and after mixing and incubation at 37°C for 45 minutes, 500µL of LC-MS grade water and 1 mL n-hexane were added to each tube. The tubes then were vigorously shaken for 15 minutes and centrifuged at 800g for 3 minutes. The organic layers were then transferred to 1.1mL polypropylene vials, placed in a Savant SpeedVac Plus Model Sc210A (Thermo Life Sciences) at 60°C, and evaporated to dryness. The dried extracts were reconstituted with 200 µL of the mobile phase mixture (LC-MS grade Water: methanol/acetonitrile (2:1), 2:8) mixed for 1 min, and then transferred to autosampler tray set at 15°C temperature. The autosampler injected a 10µL aliquot of each extract onto the analytical column (**Figure 44**). To correct 4β-hydroxycholesterol levels, total cholesterol was measured on Roche Modular P800 analyzer.

6.3.2.5 Chromatographic Condition:

Validated LC-MS/MS method was used for the analysis of 4β-hydroxycholesterol in plasma. The Acquity UPLC® BEH phenyl, 1.7µm, 2.1×100 mm column (Waters, Etten-Leur, The Netherlands) was used for the separation of 4β-hydroxycholesterol and internal standard at temperature of 27°C. The mobile phase was pumped isocratically at a flow rate of 0.3 mL/min and consisted of a mixture of mobile phase A (LC-MS water + 1 mL/L formic acid) and mobile phase B (Methanol/Acetonitrile (2:1 (w/w)) at a ratio of 21:79 respectively. The sample injection volume was 10 µL. Tandem mass spectrometric detection and quantification was performed in the positive electrospray ionization mode using multiple reaction monitoring (MRM) mode. Mass transitions selected were m/z 635/146 and 613/490 for 4β-OHC quantifier and qualifier ions,

respectively and m/z 642/146 for 4β-OHC-d7. Ms Settings were optimized manually. The source temperature was set at 130°C and the desolvation temperature was maintained at 350°C. The desolvation gas flow and cone gas flow were set at 800L/hr and 150L/hr, respectively. The capillary voltage and cone voltage were kept at 1.5kV and 38V, respectively for all compounds. The dwell times were set at 100 msec. The collision cell was set at 7.42 e-3 mbar with argon gas. The collision energy was 25eV for both 4β-OHC and 4β-OHC-d7. A Windows PC running MassLynx software was used to control the LC-MS/MS, record the output from the detector and integrate the peak areas of the drug and the internal standard. For quantitative calculation of 4β-hydroxycholesterol, an excel sheet was generated using 4β- OHC to 4β- OHC-d7 peak area ratio versus the nominal 4β- OHC concentrations with linear regression weighting 1/x (**Figure 44**).

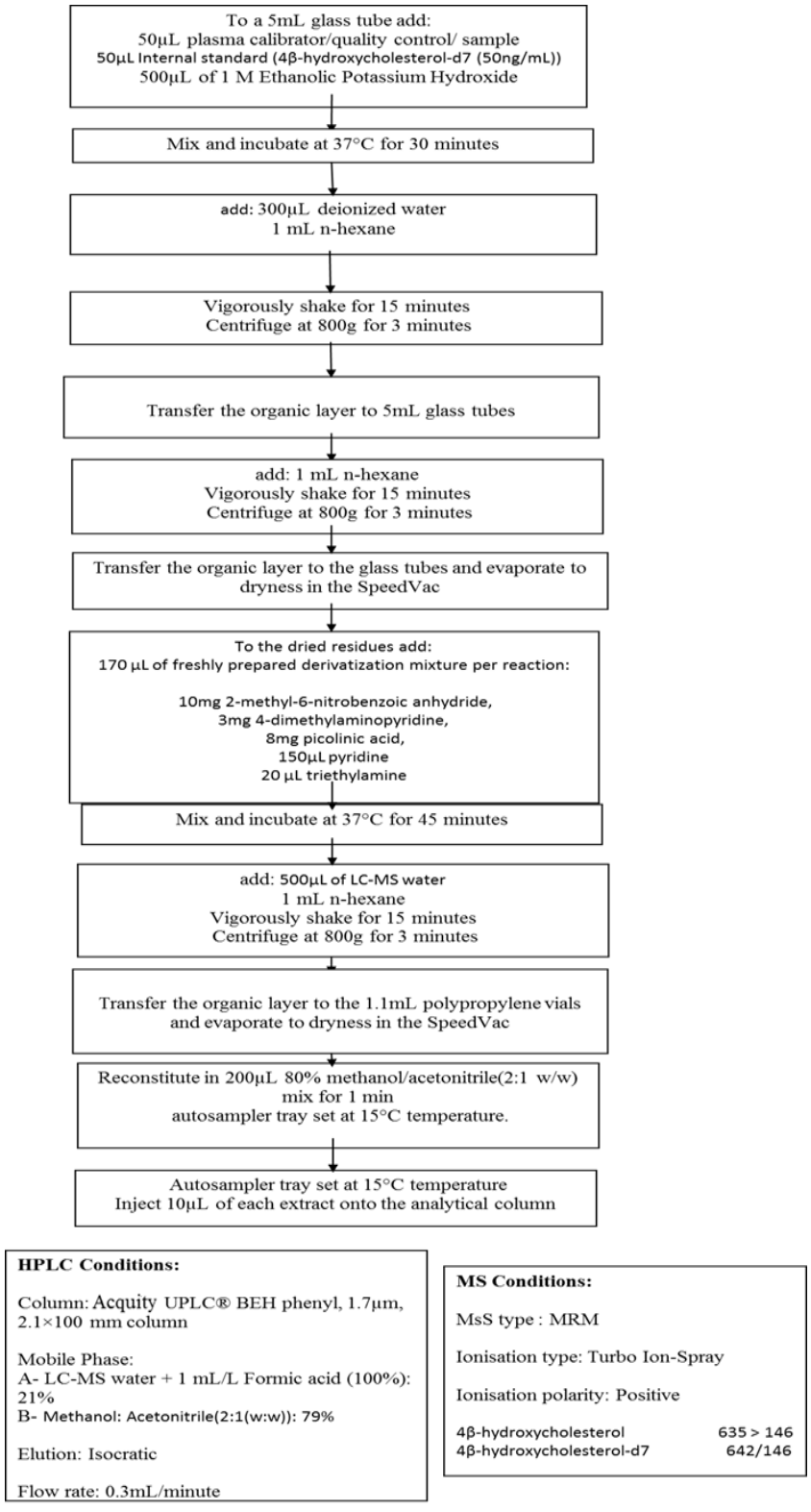


Figure 44: Schematic Diagram of the 4β-hydroxycholesterol Extraction Procedure.

6.3.3 Tacrolimus Blood Concentration Measurement

Blood concentration of tacrolimus was determined using the liquid chromatography-mass spectrometry (LC - MS - MS) method according to the procedures that described previously (**Chapter 4**). Both the within-day and between-day accuracy values were within 93.1-109.4% and 95.1-105.3%, respectively. The lower limit of quantitation was 250ng/L.

6.3.4 Determination of CYP3A5 Genotype.

DNA was extracted from peripheral blood samples using QIAamp DNA Blood Mini Kit (QIAGEN®, West Sussex, UK). *CYP3A5*1/*3* polymorphism was genotyped using real time polymerase chain reaction (PCR), a LightCycler based technique, as described in detail in **Chapter 4**.

6.3.5 Statistical Analysis

4β-hydroxycholesterol and cholesterol individual plasma concentrations were determined for each genotype. Statistical analysis was performed using Minitab statistical software (Minitab17) to assess the statistical significance of differences in 4β-hydroxycholesterol and 4β-hydroxycholesterol: cholesterol ratios in tacrolimus kinetics between different genotype groups. The log-transformed data was analysed using analysis of variance (ANOVA). Data are expressed as mean ± standard deviation (S.D.).

6.4 Results

6.4.1 Demographic Data.

Table 43 shows the demographic data of the 59 stable kidney transplant recipients. Twelve patients were homozygous for *CYP3A5*1*, 31 were homozygous for *CYP3A5*3* and 16 were heterozygous. The *CYP3A5*3* genotype frequency of the recipients deviated from Hardy-Weinberg equilibrium ($p < 0.05$). Of the 59 subjects, 36 patients were treated with prednisolone. Regarding ethnic origin, 36 patients were from white background, 12 Asian and 11 Black.

Table 43: Demographic Characteristics of Kidney Transplant Recipients.

Characteristics	Results
Age (yr), mean (SD)	54.1 (12.9)
Body weight (kg), mean (SD)	76.7 (15.3)
Male gender, n (%)	40 (68%)
Ethnic group, n (%)	
White / Black/ Asian	36/11/12
Time since transplantation (years)	
Median (range)	2.1 (0.3-22.8)
Diabetes	12 (20%)
Haemoglobin (Hb, g/L)	130.8 ± 16.1
Haematocrit	0.4 ± 0.05
Immunosuppression at baseline:	
Tacrolimus, n (%)	
Prograf®/ Adoport®	45 (76%) /14 (24%)
Corticosteroids, n (%)	36 (61%)
***CYP3A5* polymorphism**	
*CYP3A5*1/*1*	12
*CYP3A5*1/*3*	16
*CYP3A5*3/*3*	31
***CYP3A4* polymorphism**	
*CYP3A4*22 CC*	56
*CYP3A4*22 CT*	3
***ABCB1* polymorphism**	
ABCB1 CC	14
ABCB1 CT/TT	45
***POR*28* polymorphism**	
*POR*28 CC*	28
*POR*28 CT/TT*	31

6.4.2 CYP3A5 Genotype Relationship with 4β-OHC and 4β-OHC/C Ratio

The plasma 4β-OHC concentrations and 4ß-OHC/C ratios showed a log-normal distribution, and the data were therefore log-transformed before analysis. As shown in **Table 44,** a significant difference in 4β-OHC and 4β-OHC/C ratios was observed between *CYP3A5*1/*1* and **1/*3* in comparison with the **3/*3* carriers ($p<0.01$). However, there was no corresponding significant difference in 4β-OHC and 4β-OHC/C ratios between *CYP3A5*1/*1* and **1/*3* genotypes ($p>0.05$). Plasma concentrations of 4β-hydroxycholesterol were 32.1 ± 12.6, 28.2 ± 16.5 and 20.9 ± 8.7 µg/L in patients with *CYP3A5*1/*1*, **1/*3* and **3/*3* genotypes, respectively (**Figure 45 A).** The same trend was found in 4β-OHC/C ratio. The mean 4β-OHC/C for *CYP3A5*1/*1*, **1/*3* compared to **3/*3* genotypes were 7.64 ± 2.3, 7.09 ± 4.1 and 5.03 ± 2.0, respectively ($p<0.01$ by ANOVA, general linear model) and no difference was observed between *CYP3A5*1* variant allele carriers (**Figure 45 B).** Based on the above mentioned results patients were divided into two groups CYP3A5 expressers (*CYP3A5*1/*1* and **1/*3*) and CYP3A5 non-expressers (*CYP3A5*3/*3*). Plasma concentrations of 4β-hydroxycholesterol were 29.9 ±14.8 and 20.9 ± 8.7 µg/L in recipients with *CYP3A5*1* allele (n =28) and recipients with **3/*3* (n = 31) genotype, respectively ($P<0.01$, **Figure 45 C**)**.** Additionally, there was a significant increase in 4β-OHC/C ratio in the CYP3A5 expresser group (**1/*3* and **1/*1*, n = 28) in comparison with the CYP3A5 non-expresser group (**3/*3*, n = 31). The mean 4β-OHC/C ratio for *CYP3A5*1* carriers and **3/*3* genotype were 7.3 ± 3.4 and 5.0 ± 2.0 µg/L, respectively ($P<0.001$**, Figure 45 D**)**.**

Table 44: Plasma Concentrations of 4β-hydroxycholesterol in Stable Kidney Transplant Recipients with Different Ethnic Backgrounds and *CYP3A5*3* Genotypes.

	4β-OHC (µg/L)	p-value	4β-OHC/C Ratio	p-value
CYP3A5*3				
All patients				
**1/*1* (n = 12)	32.1 ± 12.6		7.6 ± 2.3	
**1/*3* (n = 16)	28.2 ± 16.5	0.25	7.1 ± 4.1	0.33
**3/*3* (n = 31)	20.9 ± 8.7	0.01	5.0 ± 2.0	0.007
**1/*1 + *1/*3* (n =28)	29.9 ±14.8		7.3 ± 3.4	
**3/*3* (n =31)	20.9 ± 8.7	0.001	5.0 ± 2.0	<0.001
Non-black patients				
**1/*1 + *1/*3* (n = 3)	25.3 ± 7.0		6.4 ± 2.1	
**3/*3* (n = 14)	20.9 ± 8.7	0.02	5.0 ± 2.0	<0.01
Black patients				
**1/*1* (n = 9)	34.0 ± 13.6		8.0 ± 2.6	
**1/*3* (n = 2)	50.6 ± 47.8	0.71	12.1±11.3	0.70
Ethnicity				
Black (n = 11)	36.9 ± 6.2		8.8 ± 4.6	
White (n = 36)	22.7 ± 1.4	<0.01	5.4± 1.9	<0.01
Asian (n = 12)	21.5 ± 2.6	<0.05	5.8 ± 2.7	<0.05
Ethnicity in *CYP3A5 Expressers (*1*1/*1*3)*				
Black (n = 9)	37.0 ± 20.6		8.8 ± 4.6	
White (n = 11)	26.0 ± 4.7	0.23	6.1 ± 1.3	0.13
Asian (n = 8)	24.5 ± 9.3	0.07	6.7 ± 2.8	0.20

The results were compared with the top group in each sub-table. Values were compared using ANOVA (General linear model). Data are shown as mean ± SD.

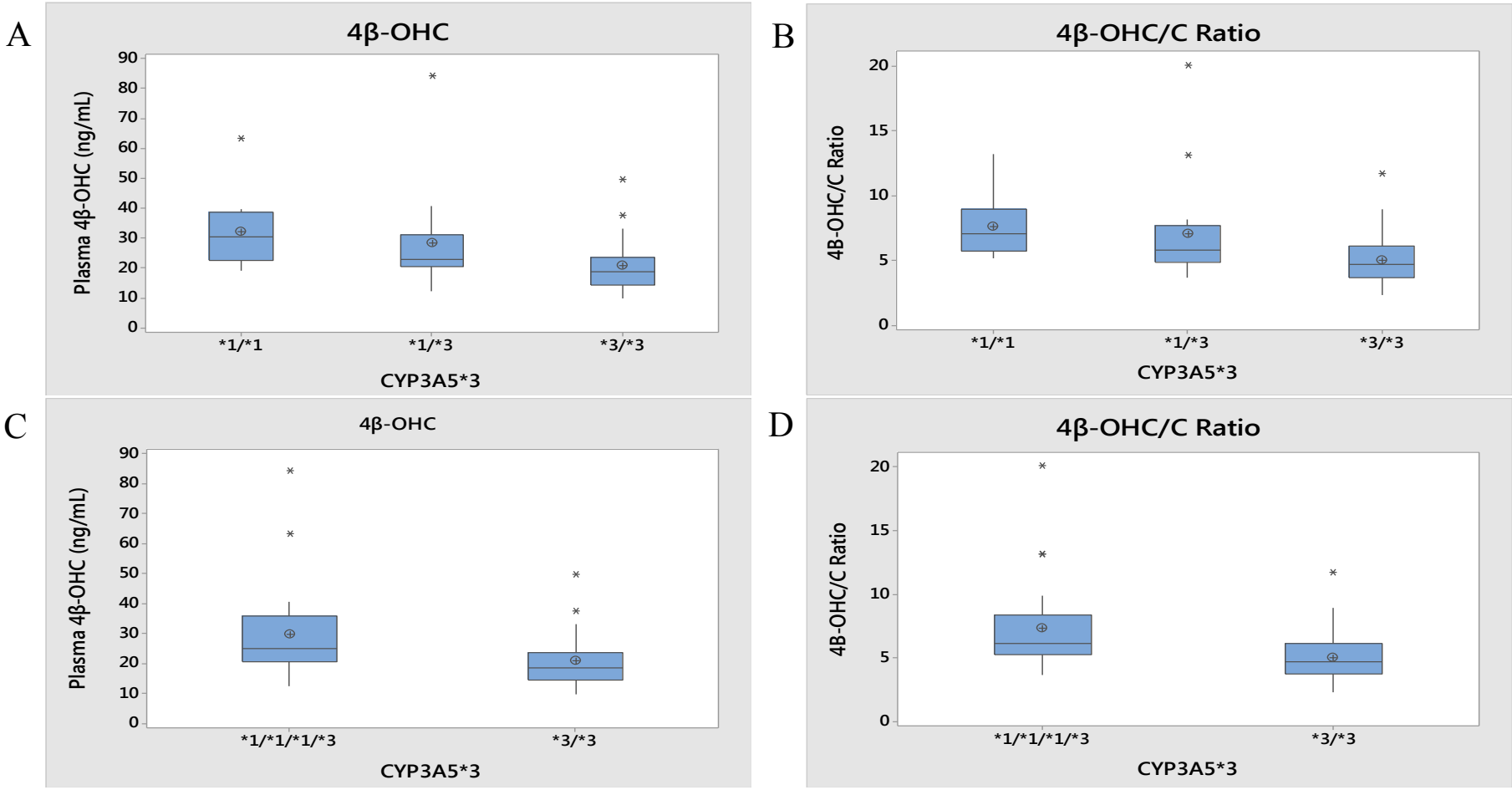


Figure 45: The Relationship of Different *CYP3A5*3* Genotypes with 4β-hydroxycholesterol Plasma Concentrations (A & C) and 4β-hydroxycholesterol / Cholesterol Ratio (B & D) in Stable Kidney Transplant Recipients. Boxes represent the interquartile range, line in the box represents the median value, symbol in the box represents the mean value, outer lines represent the minimal and maximal value and the values beyond the whiskers (asterisks) are outliers.

By studying the relationship of ethnicity with the 4ß-OHC plasma concentration, it was found that 4β-OHC/C ratio was higher in Black subjects than White and Asian ethnic groups. The mean plasma concentrations of 4β-OHC in White, Asian and Black patients were 22.7 ± 1.4, 21.5 ± 2.6 and 36.9 ± 6.2 µg/L, respectively. Black subjects had significantly higher 4β-OHC concentrations in comparison to Whites (P<0.01) and Asian (P<0.05) and no significant difference was observed between White and Asian subjects (**Table 44& Figure 46).**

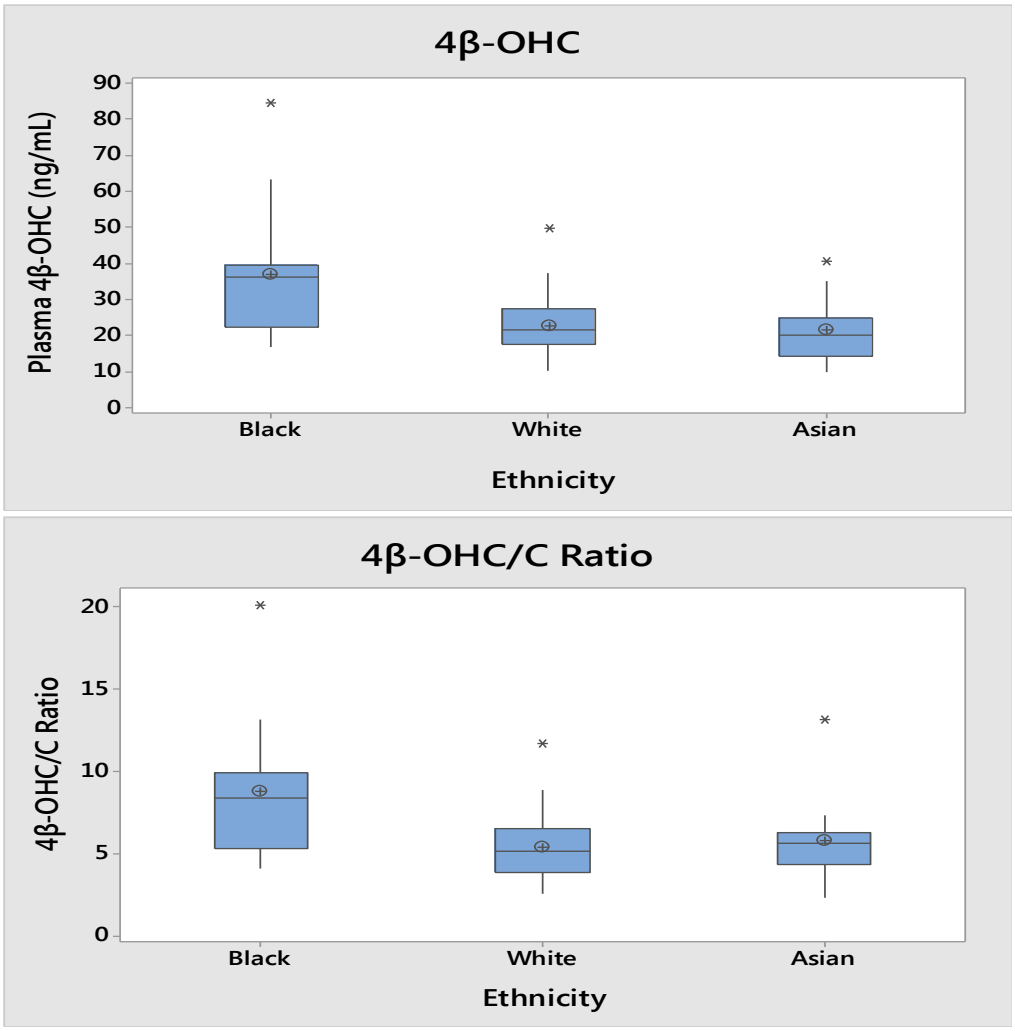


Figure 46: Ethnicity Relationship with 4β-OHC Concentration and 4β-OHC/C Ratio. Boxes represent the interquartile range, line in the box represents the median value, symbol in the box represents the mean value, outer lines represent the minimal and maximal value and the values beyond the whiskers (asterisks) are outliers.

The analysis was repeated excluding black patients. Again, CYP3A5 expressers *(*1/*1*, n=3 and **1/*3*, n = 14) demonstrated a significant increase in 4β-OHC/C ratio compared with the CYP3A5 non-expressers (**3/*3*, n = 31). The mean 4β-OHC/C ratio was 6.4 ± 2.1 for *CYP3A5*1* carriers and 5.0 ± 2.0 for CYP3A5 non-expresser (P<0.05, **Figure 47**). In addition, we observed no differences in the mean of 4β-OHC/C ratio between *CYP3A5*1/*1* (n=9) versus *CYP3A5*1/*3* (n=2) genotypes in black patients (P = 0.7; **Table 44**). By repeating the analysis in CYP3A5 expressers only, no significant difference was observed between Black (n=11), white (n=9) and Asian (n=8) subjects (**Table 44).**

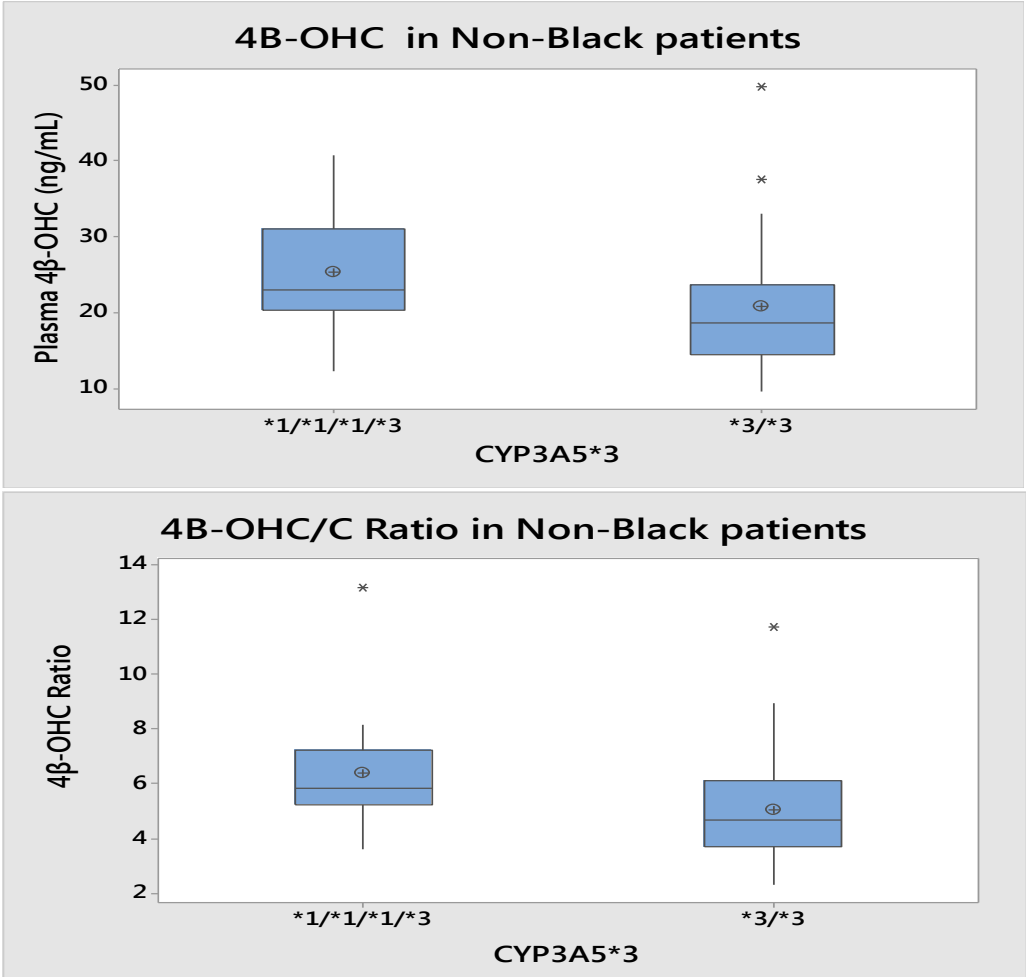


Figure 47: 4β-hydroxycholesterol Plasma Concentrations in Stable Non-black Kidney Transplant Recipients with Different *CYP3A5*3* Genotypes. Boxes represent the interquartile range, line in the box represents the median value, symbol in the box represents the mean value, outer lines represent the minimal and maximal value and the values beyond the whiskers (asterisks) are outliers.

6.4.3 CYP3A5 Genotype Association with Tacrolimus Pharmacokinetics and Dose Requirement

As shown in **Table 45**, highly significant differences in tacrolimus exposure and dose requirements were observed between CYP3A5 expresser and CYP3A5 non-expresser patients. *CYP3A5*1* allele carriers have a significant decrease in dose-normalized AUC_{0-24}, C_{max} and C_0 compared to *CYP3A5*3* carriers (P<0.001). On the other hand, there was a significant increase in tacrolimus dose requirement between the two genotype groups (P<0.001, **Figure 48**). Similar results were obtained from a larger cohort of patients in **Chapter 4**.

Table 45: *CYP3A5*3* Genotype Relationship with Dose-Normalized Tacrolimus PK Parameters and Dose Requirement.

PK-parameter	CYP3A5 Expressers (**1/*1* & **1/*3*) (n= 28)	CYP3A5 Non-expressers (**3/*3*) (n= 31)	p-value
Dose (mg/Kg/day)	0.11 ± 0.05	0.05 ± 0.02	< 0.001
C_{max} (µg/L/mg/Kg)	20.4 ± 12.2	34.5 ± 14.3	< 0.001
AUC_{0-24} (µg*h/L/mg/Kg)	227 ± 157	462 ± 215	< 0.001
C_0 (µg/L/mg/Kg)	7.4 ± 5.8	16.0 ± 9.3	< 0.001

Values were compared using ANOVA (General linear model).
Data are shown as mean ± SD.

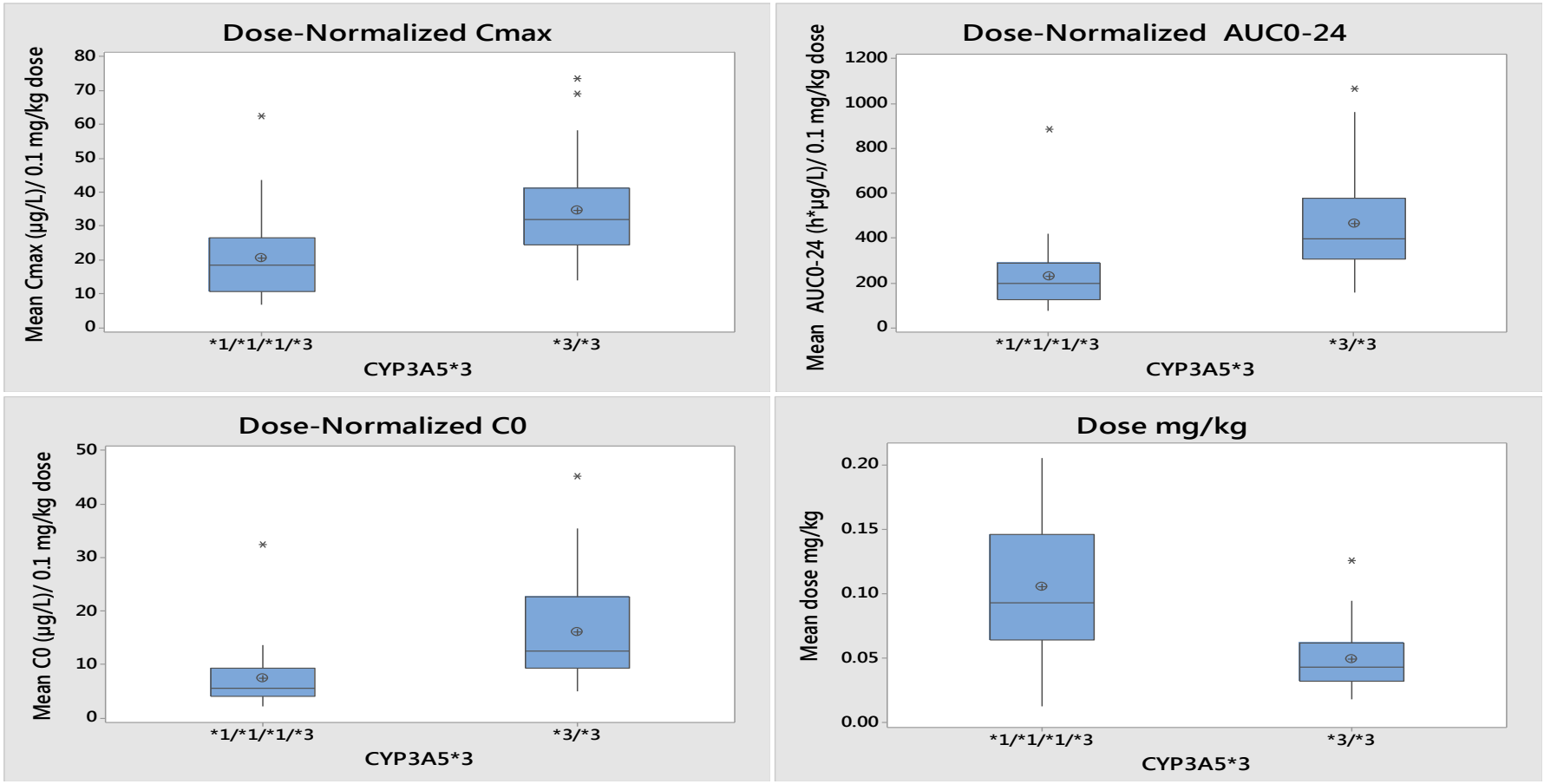


Figure 48: *CYP3A5*3* Genotype Relationship with Tacrolimus Pharmacokinetic Parameters and Dose Requirement. Boxes represent the interquartile range, line in the box represents the median value, symbol in the box represents the mean value, outer lines represent the minimal and maximal value and the values beyond the whiskers (asterisks) are outliers.

6.4.4 The Relationship of 4β-OHC with Tacrolimus Exposure and Dose Requirement

A significant positive correlation was observed between log 4β-OHC/C ratio and tacrolimus dose (r = 0.45, p < 0.001). On the other hand, A significant negative correlation was observed between log 4β-OHC/C ratio and tacrolimus pharmacokinetic parameters**,** dose-normalized C_{max} (r = -0.35, p < 0.01), dose-normalized AUC_{0-24} (r = -0.41, p < 0.01) and dose-normalized C_0 (r = -0.41, p < 0.01). The relationships of tacrolimus dose and PK parameters with 4β-OHC/C ratio in stable kidney transplant recipients are shown in **Figure 49**. Factors associated with dose requirements of tacrolimus were studied using univariate regression analysis. The P value was highly significant with ethnicity, *CYP3A5*3* genotype and log-transformed 4β-OHC/C ratio (p < 0.001 for each) and *ABCB1 3435* genotype (p<0.01). The P values for sex and haematocrit were < 0.05 and it was 0.071 for age and 0.09 for *CYP3A4*22* genotype.

Multiple regression analysis by stepwise selection; alpha to enter or remove was 0.15; identified 4β-OHC/C ratio, *CYP3A5*3* genotype, age, ethnicity, haematocrit and *CYP3A4*22* genotype, as independent variables associated with tacrolimus dose and this explains 62.5% of the variability in tacrolimus dose. The contributions of the individual variables are shown in **Table 46**. After adjusting for these independent predictors of tacrolimus dose, *CYP3A5**3 genotype had a strong association with tacrolimus dose (p <0.001) and accounts for 34.1% of the variability in tacrolimus dose requirement. Patient age became strongly associated with tacrolimus dose (P <0.05, **Table 46**). Furthermore, when we repeat the multiple regression analysis by using the *CYP3A5*3/ABCB1*combined genotype instead of *CYP3A5*3* and *ABCB1* separately, the percentage of tacrolimus dose variability explained by these variables increased to be 63.4%, **Table 46**. After adjusting for these independent predictors of tacrolimus dose, patient age became strongly associated with tacrolimus dose (P <0.05). The log-transformed 4β-OHC/C Ratio, *CYP3A5/ABCB1* Genotype and haematocrit were responsible for 23.2%, 19.7% and 10.4%, respectively, of the variability in tacrolimus dose requirements. The contributions of the individual variables are shown in **Table 46**.

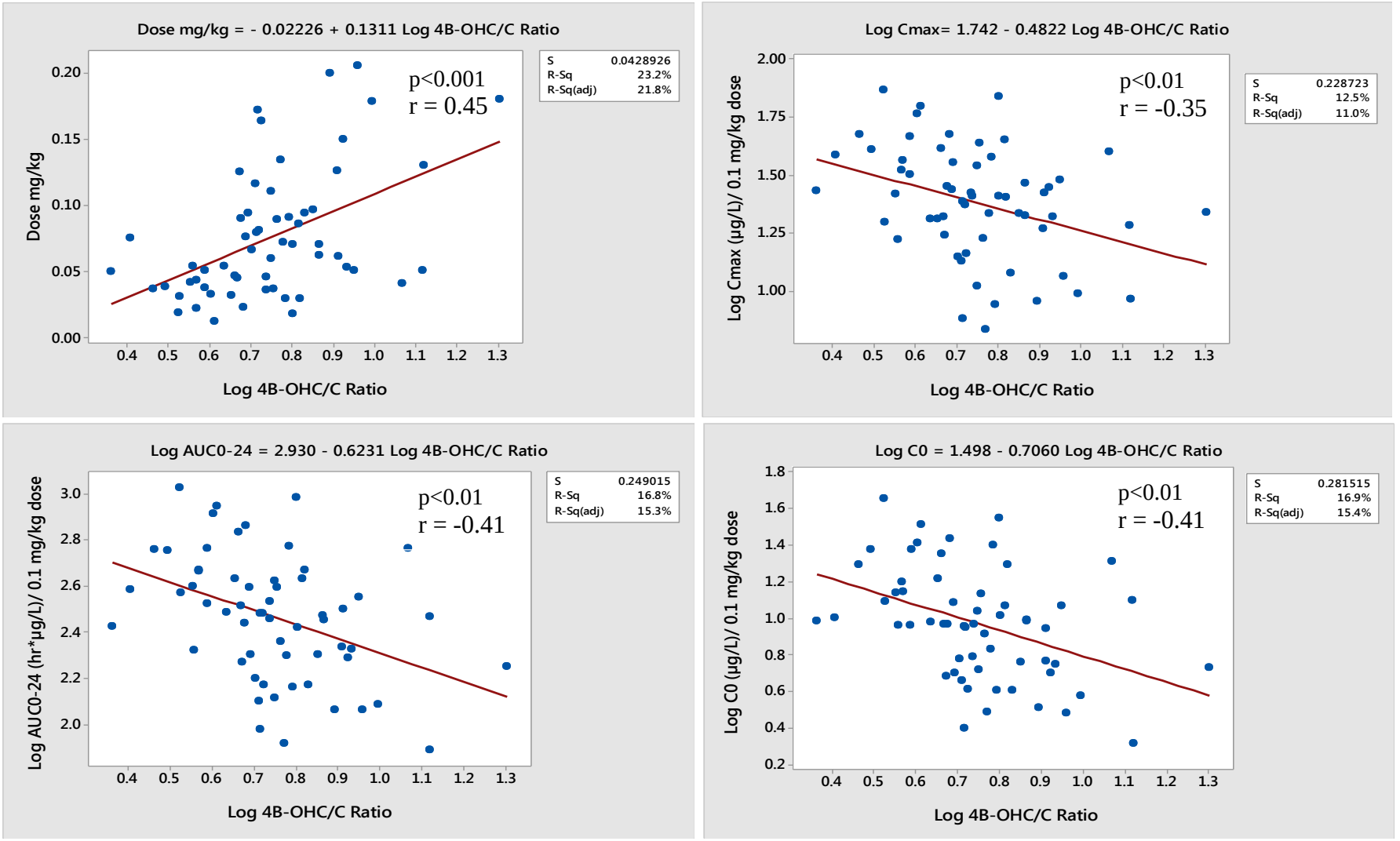


Figure 49: The Fitted Line Plot for 4β-OHC/C Ratio Effect on Dose-Normalized Tacrolimus Pharmacokinetic Parameters and Dose Requirement. Values were compared using regression analysis.

Table 46: Stepwise Regression Equation of Tacrolimus Dose (mg/kg) Requirement after Renal Transplantation.

Stepwise regression equation	R^2	Independent variables
A. *CYP3A5* and *ABCB1* separate genotypes **Dose (mg/kg)** = 0.1209 + 0.0838*Log 4β-OHC/C Ratio - 0.000880*Age - 0.248* Haematocrit - 0.0391(if Asian) - 0.0104 (if White) + 0.0374 (if CYP3A5 **1/*1* & **1/*3*) + 0.0369 (if *CYP3A4*22 CC*).	62.5%	Age (p = 0.021, R^2 = 2.5%) Ethnicity (p = 0.013, R^2 = 8.3%) Haematocrit (p = 0.018, R^2 = 9.2%) *CYP3A4*22* genotype (p = 0.084, R^2 = 2.3%) *CYP3A5*3* genotype (p = 0.001, R^2 = 34.1%) Log 4β-OHC/C Ratio (p = 0.006, R^2 = 6.0%)
B. *CYP3A5* and *ABCB1* combined genotypes **Dose (mg/kg)** = 0.1112 + 0.0835*Log 4β-OHC/C Ratio - 0.000951*Age - 0.235* Haematocrit - 0.0322 (if Asian) - 0.0032 (if White) + 0.0399 (if *CYP3A4*22* CC) + 0.045 (if CYP3A5**1/*1&*1/*3/ABCB1CC*) + 0.0342 (if CYP3A5**1/*1&*1/*3/ABCB1CT/TT)* - 0.0207 (if CYP3A5 **3/*3/ABCB1CC*).	63.4%	Age (p = 0.016, R^2 = 3.1%) Ethnicity (p = 0.039, R^2 = 4.5%) Haematocrit (p = 0.028, R^2 = 10.4%) *CYP3A4*22* genotype (p = 0.067, R^2 = 2.7%) *CYP3A5/ABCB1* Genotype (p = 0.011, R^2 = 19.7%) Log 4β-OHC/C Ratio (p = 0.007, R^2 = 23.2%)

R^2 gives the % variability explained by the independent variables in the multiple regression analysis model.

6.5 Discussion

4β-hydroxycholesterol has been reported as a marker of CYP3A activity and it is suitable for CYP3A activity evaluation in stable kidney transplant recipients; 4β-OHC was stable 90 days after kidney transplantation (Suzuki et al., 2013b). Additionally, tacrolimus is known as CYP3A5 substrate and CYP3A5 polymorphisms influence tacrolimus trough blood concentration and dose requirements in stable kidney transplant patients (Macphee et al., 2005). In this study, we investigated the changes in tacrolimus dose in relation to CYP3A activity and 4β-OHC/C ratio in stable kidney transplant patients.

First, our study showed that there was a reduction in 4β-OHC concentration with increasing number of the *CYP3A5*3* allele. However, this decrease was not significant between CYP3A5 expressers subgroups (**1/*1* and **1/*3*), this was different from the previous reports which showed that 4β-OHC concentration decreased significantly with increasing number of the *CYP3A5*3* allele (Diczfalusy et al., 2008, Suzuki et al., 2014). After excluding black patients from the analysis, we still found a significant difference between CYP3A5 expressers and non-expressers. *CYP3A4*22* was not included in this study because the low frequency of this genotypes in our population. In addition, *ABCB1* polymorphism had a significant effect on 4β-OHC/C ratio. Moreover, *POR*28* had no significant effect on 4β-OHC/C ratio.

Second, we found that Black subjects had significantly higher 4β-OHC concentrations in comparison to White and Asian subjects. The majority of our population were of Caucasian ethnic background and mainly males. This is different from previously published report showing that Tanzanian subjects (Black-Africans) had the lowest mean 4β-hydroxycholesterol concentration followed by Swedes (Caucasians) and Koreans (Asians) who had the highest 4β-hydroxycholesterol concentration (Diczfalusy et al., 2008). The discrepancy between the results may be due to the genetic difference in both studies' populations. In our study, Black patients were North African, whereas in the other study they were Western African. They also found a significant difference in the mean 4β-hydroxycholesterol concentration between the three ethnic groups in women.

In contrast, no difference was observed between Swedish (Caucasians) and Korean (Asians) men (Diczfalusy et al., 2008). However, it is likely that the ethnic difference is simply a reflection of the distribution of the CYP3A5 genotype. After excluding CYP3A5 non-expressers from the analysis, we didn't find any significant difference in 4β-OHC concentration and 4β-OHC/C ratio among the Black, White and Asian ethnic groups.

Third, we confirmed that *CYP3A5*3* allele had a significant impact on tacrolimus disposition and dose requirement in this patient sample. Patients carrying at least one *CYP3A5*1* allele had a significantly lower dose-normalized tacrolimus C_{max}, AUC_{0-24} and C_0 compared to *CYP3A5*3/*3* and required a higher tacrolimus dose than CYP3A5 non-expressers (MacPhee et al., 2004, Tada et al., 2005, Cusinato et al., 2014).

Fourth, a significant correlation was observed between plasma concentrations of 4β-hydroxycholesterol or 4β-OHC/C ratio and tacrolimus dose requirement, suggesting that the higher 4β-hydroxycholesterol levels, indicating high CYP3A activity and increase tacrolimus dose requirement in stable kidney transplant recipients.

Finally, multiple regression analysis by stepwise selection identified *CYP3A5*3* genotype (or *CYP3A5*3/ ABCB1* combined genotype), log 4β-OHC/C ratio, age, ethnicity, haematocrit and *CYP3A4*22* genotype as independent variables associated with the required tacrolimus dose. Our findings suggest that these independent variables may explain 62.48% to 63.37% of the between-patient variability in tacrolimus dose. This is the first report of plasma 4β-hydroxycholesterol concentration as a biomarker of CYP3A activity in addition to *CYP3A5*3* polymorphism to predict the required tacrolimus dose in stable kidney transplant recipients. These findings may help to explain the variability in tacrolimus dose between individuals which cannot be explained only by the genetic factors. Further studies are required to validate the clinical importance and utility of these findings.

Chapter 7. Tacrolimus Within-Patient Variability: the Impact of Conversion from Immediate (Prograf® or Adoport®) to Prolonged Release (Advagraf®) Tacrolimus Formulations in Stable Renal Transplant Patients.

7.1 Objective of the Study:

1. To study the effect of switching stable renal transplant patients from a twice daily formulation of tacrolimus (Prograf® or Adoport®) to once daily formulation (Advagraf®) on within patient variability in pre-dose blood tacrolimus concentration.
2. To investigate tacrolimus within-patient variability correlation to *CYP3A5* genotype in stable kidney transplant recipients for twice a day tacrolimus (Prograf® or Adoport®) and once a day tacrolimus, Advagraf®.

7.2 Materials and Methods

7.2.1 Patients and Study Design:

A retrospective, cross-sectional study was performed for 100 renal transplant recipients attending routine follow-up clinical evaluations at the kidney transplant clinic, St George's Hospital. Clinically stable patients underwent scheduled switching from twice- to once- daily tacrolimus between January 2010 and March 2014. As part of St George's Hospital routine care, patients only switched to once-daily tacrolimus if they had a stable graft function without evidence of acute rejection episodes over the previous 3 months. Patients qualified for the study if they were above 18 years old, had completed at least 3 months of transplantation, had a stable graft function for at least 6 weeks, and were treated with no more than 5 mg prednisolone daily at the beginning of the study period and were followed-up for at least 12 months. Exclusion criteria included patients under 18 years old or treated with more than 5 mg prednisolone daily. Conversion from tacrolimus twice-daily to once-daily was made on a 1mg: 1mg basis of the total daily dose with target tacrolimus trough concentration 5-8 µg/L) during both

periods (pre-conversion and post-conversion). For each patient, demographic and transplant characteristics, relevant clinical data, all tacrolimus dose changes, all tacrolimus trough blood concentrations and co-medications were obtained retrospectively from medical records. Estimated glomerular filtration rate (eGFR) was calculated using Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) Equation among OD-Tac converted patients (Levey et al., 2009).

7.2.2 Within-Patient Variability (WPV) Calculation:

During the study period, Tacrolimus trough concentrations obtained as a component of routine care were determined using the Dade-Behring immunoassay (Thermo Scientific, 2011). The limit of detection was 2.0 ng /mL and the total imprecision of the method was < 16.5% coefficient of variation. The accuracy and precision were evaluated by the International Tacrolimus Proficiency Testing Scheme. All tacrolimus concentration measurements and time of blood sampling were obtained from the electronic hospital database. Dose-normalized trough concentrations were calculated to obtain comparable values that are independent of the individual dose and body weight. Analysis of dose-normalized tacrolimus trough blood concentrations (Tac C_0) was made during periods of stable tacrolimus doses and over the periods before and after conversion. The duration of follow-up was 12 months after conversion. For all the patients, at least three daily tacrolimus doses and their corresponding trough blood concentrations were collected from the electronic patient records at St George's Hospital. The mean number of samples per patient was 6.3 ± 2.7 for periods of tacrolimus stable dose. The mean number of samples per patient was 13.4 ± 10.6 and 14.3 ± 6.5 for twice- and once-daily tacrolimus, respectively, over the periods before and after conversion, including the whole period and not just periods of stable dosing. Within-patient variability (WPV) of dose-normalized tacrolimus trough blood concentrations was calculated using the following equation:

$$\%CV = (SD \times 100) / X$$

Where, **SD** is the standard deviation and **X** is the mean value of all the analysed tacrolimus concentration samples.

Patients were characterized as having a high or low WPV using the median value of WPV as the cutoff value, the approach used previously by Borra et al. (2010). Analysis of WPV of tacrolimus trough blood concentrations (Tac C_0) after conversion to Advagraf® was made during periods of stable tacrolimus doses and additionally, another analysis was made over the whole periods before and after conversion. To investigate the relation between within-patient variability and *CYP3A5* polymorphisms in both tacrolimus formulations, all patients who participated in our previous studies and whose *CYP3A5* genotype was known (n=72) were included in this study.

7.2.3 Statistical Analysis

The WPV was calculated using Microsoft Excel 2010. Statistical analysis was performed using Minitab statistical software (Minitab 17). Data on patients' baseline characteristics were expressed as mean or range. Differences in WPV between the treatment periods were assessed using ANOVA. The log-transformed data were analysed using analysis of variance (ANOVA) with factors for genotype group and treatment. Kruskal–Wallis test was also used to compare medians of CV% between different treatments and *CYP3A5* genotypes when the values were non-normally distributed.

7.3 Results

7.3.1 Patients' Characteristics at Baseline (Pre-Conversion Patient Characteristics)

This study of tacrolimus formulation conversion included 100 patients (60 men and 40 women) in the period January 2010 to March 2014. The baseline characteristics of the study population are shown in **Table 47**. All recipients had at least 1-year follow-up. The mean age of recipients at the time of conversion from twice-daily to once-daily tacrolimus was 52.9 ± 12.6 years. The median [range] interval post-transplant at conversion was 2.9 (0.3-22.8) years. Fifty-seven patients were cases of deceased donor kidney transplantation. The concomitant immunosuppression included prednisolone

(n=60), Mycophenolate mofetil (n=34), and azathioprine (n=21). At baseline, 63 patients (63%) had hyperlipidaemia and 27 patients (27%) had diabetes mellitus.

Table 47: Patient Demographics and Baseline Characteristics.

Characteristics	**Number of subjects (%)**
Sex: n (%)	
Male / Female	60 (60%) / 40 (40%)
Age, mean	52.9 ± 12.6
Ethnicity:	
White n (%)	59 (59%)
Black n (%)	19 (19%)
Asian n (%)	22 (22%)
Primary kidney disease: n (%)	
Hypertensive nephropathy	10 (10%)
Polycystic kidney disease	22 (22%)
Diabetic nephropathy	10 (10%)
IgA nephropathy	9 (9%)
Glomerulonephritis (other)	12 (12%)
Congenital /other	14 (14%)
Reflux disease	3 (3%)
Unknown	20 (20%)
Donor type: n (%)	
Living / Deceased	43 (43%) / 57 (57%)
Time since transplantation (years)	
Median (range)	2.9 (0.3-22.8)
Diabetes mellitus n (%)	27 (27%)
Hyperlipidemia n (%)	63 (63%)
Immunosuppression at baseline:	
TD-Tac (monotherapy)	2 (2%)
TD-Tac and MMF	17 (17%)
TD-Tac and prednisolone	43 (43%)
TD-Tac, MMF and prednisolone	17 (17%)
TD-Tac and Azathioprine	21 (21%)

7.3.2 Post-conversion Follow-Up

7.3.2.1 Graft Renal Function: One Year Follow-Up

Renal function was assessed just before and at all times after tacrolimus conversion. Mean serum creatinine, serum albumin and estimated glomerular filtration rate (eGFR) remained stable during follow up (**Table 48**). Moreover, haemoglobin concentration and systolic (SBP) and diastolic blood pressure (DBP) did not change significantly during follow up (**Table 48**). The data were obtained in 100 patients at all times

Table 48: Patient Clinical Parameters during the 12 Months after Conversion from TD-Tac to OD-Tac.

Parameters	**t=0 (n=100)**	**2 Weeks (n=82)**	**1 month (n=95)**	**3 months (n=99)**	**6 months (n=98)**	**9 months (n=97)**	**12 months (n=100)**
Serum creatinine (μmol/L)	126.1 ± 42.0	125.8 ± 39.0	123.4 ± 38.3	124.4 ± 43.4	121.7 ± 41.2	121.4 ± 39.0	123.0 ± 40.7
eGFR (mL/min)	57.5 ± 19.9	58.3 ± 20.7	58.9 ± 21.1	59.1 ± 21.6	60.5 ± 21.9	60.5 ± 21.4	60.15 ± 22.9
Albumin (g/L)	38.9 ± 3.2	39.5 ± 4.2	38.8 ± 3.5	39.5 ± 7.8	39.3 ± 7.5	38.2 ± 3.6	38.3 ± 3.4
Haemoglobin (g/L)	130.9 ± 16.9	128.7 ± 15.6	129.3 ± 15.1	131.7 ± 17.7	131.1 ± 15.3	131.0±15.1	130.8 ± 16.7
SBP (mmHg)	129.9 ± 14.1	126.5 ± 12.0	128.3 ± 12.8	126.1 ± 11.3	128.5 ± 12.5	129.9 ± 14.0	127.7 ± 13.3
DBP (mmHg)	78.6 ± 9.5	80.0 ± 8.2	78.9 ± 8.4	79.1 ± 8.7	78.1 ± 8.2	78.8 ± 9.7	79.6 ± 9.3

Data are shown as mean ± SD.

7.3.2.2 One Year Follow-Up of Tacrolimus Exposure and Dosing after Conversion to Advagraf®

For evaluation, one tacrolimus trough blood concentration and its coinciding tacrolimus dose were retrieved from the clinic database just before conversion and at 0.5, 1, 3, 6, 9 and 12 months after conversion. Before conversion, the mean morning trough blood concentration of tacrolimus (TD-Tac) was 6.6 ± 1.9 µg/L and the mean total daily dose of tacrolimus was 5.2 ± 3.4 mg. At 0.5, 1, 3 and 6 months after conversion, mean tacrolimus trough concentrations were 6.0 ± 2.3, 6.0 ± 1.8, 6.1 ±1. 9 and 5.9 ± 2.0 µg/L, respectively, with mean OD-Tac doses (mg) of 5.2 ± 3.4, 5.2 ± 3.4, 5.2 ± 3.3 and 5.2 ± 3.2, respectively. Then, in the following months, the trough blood concentrations of tacrolimus were kept at 6.2 ± 2.0 and 6.2 ± 1.8 µg/L at 9 and 12 months, respectively, with mean OD-Tac doses (mg) of 5.2 ± 3.2, 5.1 ± 3.2, respectively (**Figure 50 & Figure 51**).

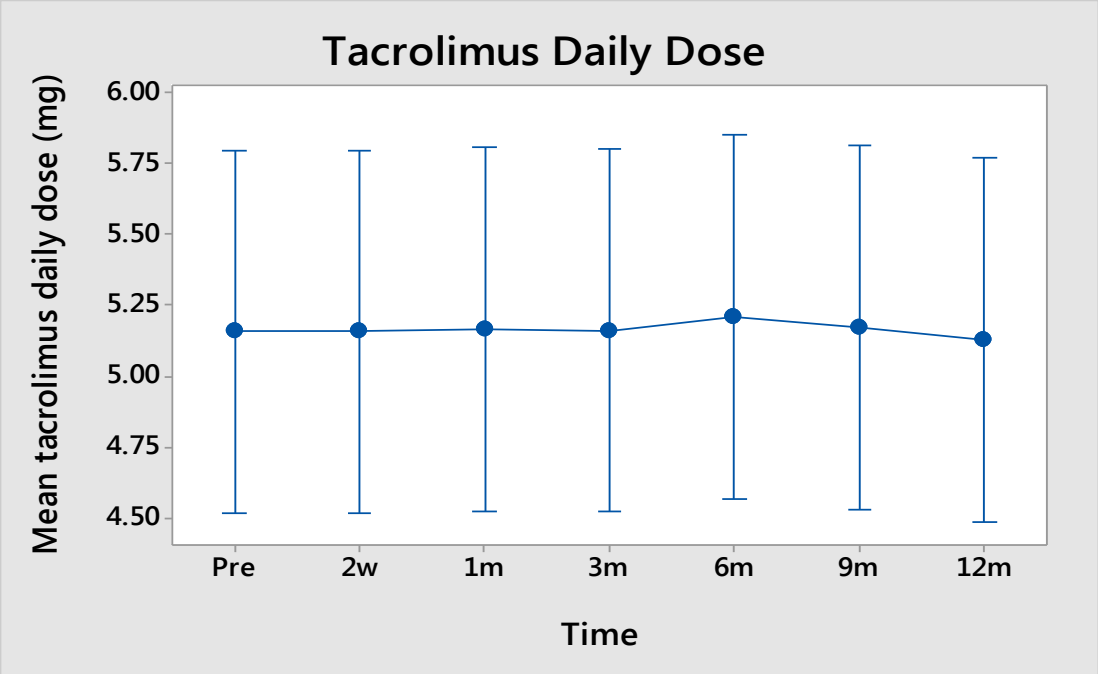


Figure 50: Tacrolimus Trough Concentrations before Conversion and in 0.5, 1, 3, 6, 9 and 12 Months after Conversion (Pre= Pre-Conversion, w=week and m=month).

Despite no change in the OD-Tac dose during follow-up period, Tac C_0 were slightly reduced in the majority of the patients with a significant decrease in tacrolimus blood concentrations after 6 months and then it reached initial levels at 9 and 12 months post-conversion. The daily doses of TD-Tac and OD-Tac doses in the analysed period are shown in **Figure 50**.

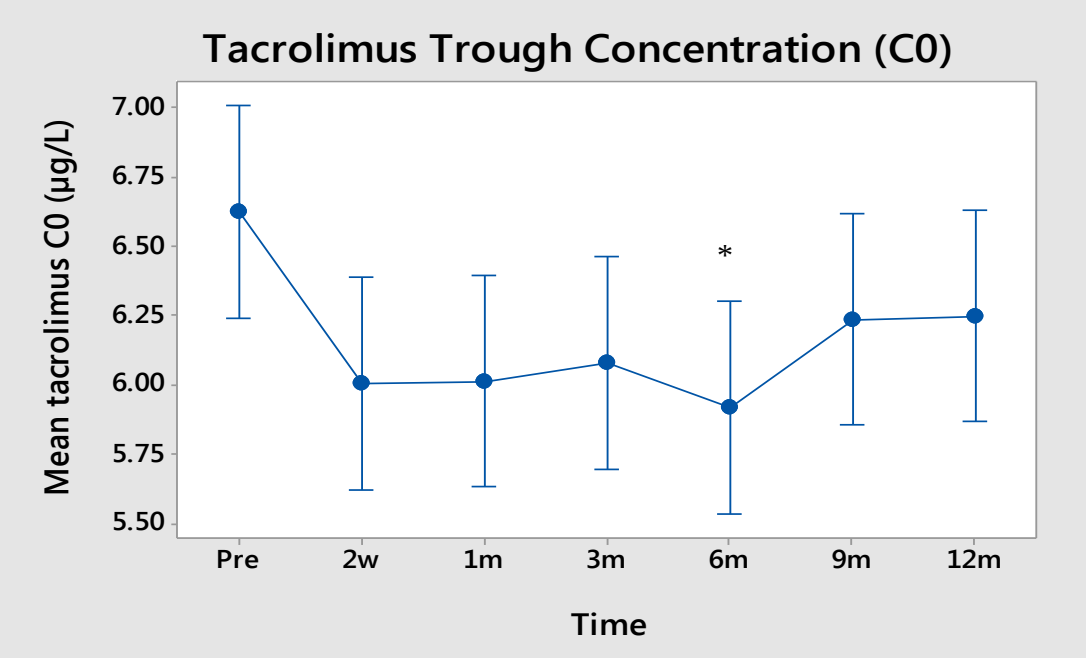


Figure 51: Tacrolimus Daily Dose before Conversion and in 0.5, 1, 3, 6, 9 and 12 Months after Conversion (Pre= Pre-Conversion, w=week and m=month). Values were compared using ANOVA (General linear model). * Significantly Different from Pre-Conversion.

7.3.3 Effect of the Switch to Advagraf® during Stable Dosing Periods and the Entire Time Pre- and Post- Conversion.

Conversion of tacrolimus from twice-daily to once-daily formulation was based on 1: 1 mg. For all patients, at least 3 samples were available for calculation of the within-patient variability of Tac C_0. Tacrolimus trough concentrations were analysed for WPV in this ethnically diverse population during periods of stable tacrolimus doses. For each patient, only one stable dosing period was used for each formulation. Additionally, the analysis was repeated for the whole periods before and after conversion. The relationship between within-patient variability and *CYP3A5* polymorphisms in both tacrolimus formulations was also studied.

7.3.3.1 Tacrolimus Exposure and Daily Dosing during Stable Dosing Periods and the Entire Time Pre- and Post- Conversion

During the period of stable tacrolimus dosing before conversion, the mean tacrolimus trough concentration (µg/L) was 7.3 ± 1.8 with a TD-Tac dose of 5.4 ± 3.3 mg. The percent change in tacrolimus dose was calculated using the last stable Prograf® or Adoport® dose before conversion versus the final stable Advagraf® doses after conversion. Advagraf® dose was modified from TD-Tac dose in 68 cases after conversion, increasing in 29/100 and decreasing in 39/100 kidney transplant recipients, based on tacrolimus trough blood concentration (Tac C_0) measurements in order to remain in therapeutic range (**Figure 52**). The mean Tac C_0 was 6.1 ± 1.0 µg/L with mean OD-Tac doses (mg) of 5.1 ± 3.1. The total daily tacrolimus dose was significantly different before and after the switch ($p < 0.05$). Moreover, the mean tacrolimus trough concentration was significantly decreased by 15% after conversion ($P < 0.001$). The dose-normalized Tac C_0 also decreased by 10 % [90%CI 11 to 19%] ($p < 0.01$; **Table 49).**

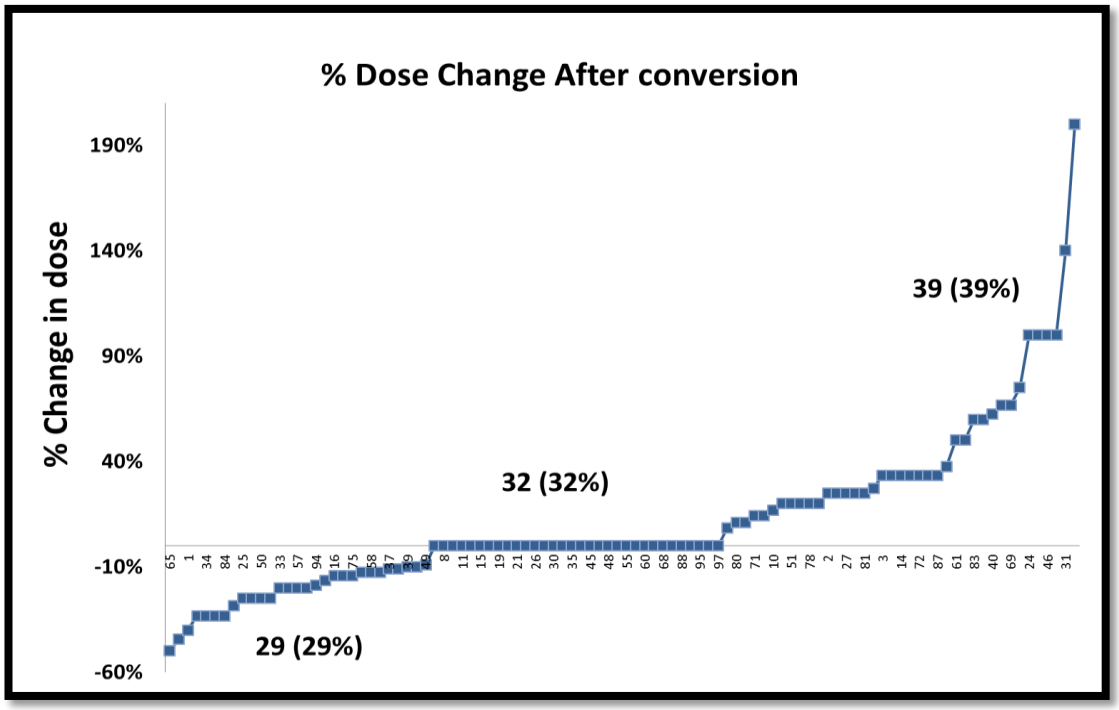


Figure 52: Percentage Change in Tacrolimus Dose after Conversion from Twice to Once-Daily Tacrolimus during Stable Dosing Periods.

Table 49: The Mean Trough Concentration (Tac C_0) and Dose of Twice-Daily (TD-Tac) and Once-Daily Tacrolimus (OD-Tac).

	TD-Tac Stable period	OD-Tac Stable period	P-value	*Pre-conversion	*Post-conversion	P-value
Tacrolimus dose (mg/day)	5.4 ± 3.3	5.1 ± 3.1	<0.05	5.5 ± 3.4	5.1 ± 3.1	<0.01
Tacrolimus dose (mg/Kg/day)	0.074 ± 0.04	0.070 ± 0.04	<0.05	0.077 ± 0.05	0.071 ± 0.04	<0.001
Tac C_0 (µg/L)	7.3 ± 1.8	6.1 ± 1.0	<0.001	7.5 ± 1.6	6.2 ± 0.91	<0.001
Tac C_0 (µg/L / mg/kg)	14.6 ± 11.0	13 ± 9.8	<0.01	14.6 ± 10.7	12.6 ± 8.7	<0.001

*Pre-conversion: the whole period before switching to OD-Tac where patients were on TD-Tac treatment. Post-conversion: the whole period after switching to OD-Tac where patients were on OD-Tac treatment. Data are shown as mean ± SD. Values were compared using ANOVA (General linear model).

The analysis was repeated to include not only the periods of stable tacrolimus dose, but also the whole period before and after conversion allowing inclusion of a greater number of data points. Again, the mean tacrolimus C_0 concentration fell from 7.5 µg/L SD 1.6 to 6.2 µg/L SD 0.1 with a 16% reduction [90%CI 13 to 19%]. The dose-normalized Tac C_0 also decreased by 11% [90%CI 7 to 14%] ($p < 0.001$). Both the daily dosage of tacrolimus and the weight-adjusted tacrolimus dose (mg/kg) were statistically significantly different before and after the conversion. The total daily Tacrolimus dose was 5.5 ± 3.4 vs. 5.1 ± 3.1 mg/day; $p < 0.01$. The percent change in tacrolimus dose was calculated using the starting versus the final Advagraf® doses. The daily Tacrolimus dose had been left unchanged in 6/100 but was increased in 39/100 and decreased in 55/100 patients. A summary of the untransformed twice- and once-daily tacrolimus mean trough blood concentrations and doses are given in **Table 49**.

7.3.3.2 Effect of Conversion to OD-Tac on Tacrolimus WPV during Stable Dosing Periods and the Entire Time Pre- and Post- Conversion

During TD-Tac treatment with stable tacrolimus dosing, the mean and median WPV of dose-normalized tacrolimus C_0 were 26.9 % SD 18.2 and 22.9% (range, 5.7%–149.2%), respectively. After conversion to OD-Tac, WPV of dose-normalized tacrolimus C_0 did not show any significant change, the mean and median WPV for TD-Tac was 24.9% SD 13.0 and 23.3% (range, 4.7%–79.2%); p = 0.39, by Balanced ANOVA and p=0.79,by Kruskal-Wallis Test, respectively, **Figure 53**.

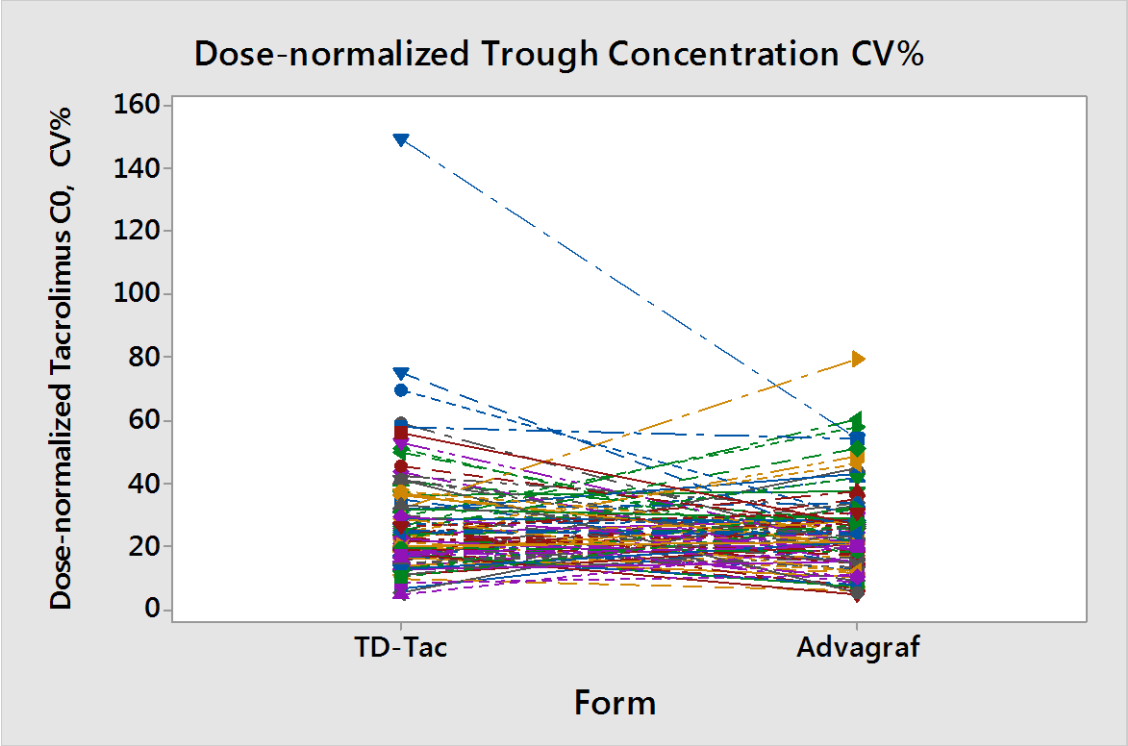


Figure 53: The Individual Change of Percent Coefficient of Variation (CV %) During Periods of Stable Tacrolimus Dose before and after Conversion from Twice-Daily Tacrolimus (TD-Tac) to Advagraf®.

After including the whole period before and after conversion, again, there was no difference in tacrolimus WPV between TD-Tac and Advagraf®. The mean and median WPV of dose-normalized tacrolimus C_0 were 29.2% SD 17.1 and 26.3% (range, 6.8%–147.5%), respectively, for TD-Tac vs. 29.2% SD 10.9 and 27.0% (range, 5.8%–60.0%), respectively for Advagraf® (p = 0.98, by Balanced ANOVA and p = 0.24, by Kruskal-Wallis Test, respectively, **Figure 54**)

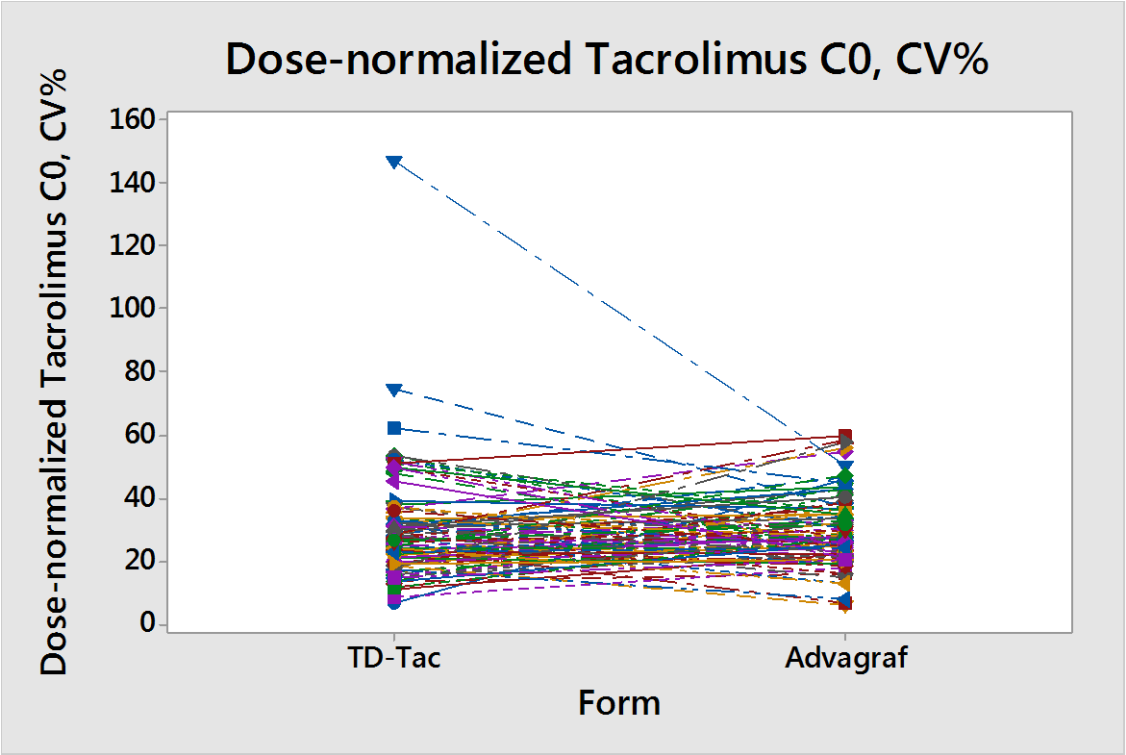


Figure 54: The Individual Change of Percent Coefficient of Variation (CV %) During the Whole Period before and after Conversion from Twice-Daily Tacrolimus (TD-Tac) to Advagraf®.

The patients then were divided into low and high tacrolimus variability groups based upon their variability using the median as the cutoff value.

The analysis was first conducted during the periods of stable tacrolimus dose. Although no significant difference in the WPV was observed in the whole population after conversion, patients with low-variability before conversion demonstrated a significant increase in WPV after conversion, WPV increased from 16.2 ± 4.6% and 17.4% (range,4.7% – 22.9%) to 21.6 ± 9.8% and 21.3% (range, 4.7% – 48.6%, p <0.001 for each). The reverse was observed in high-variability group, WPV decreased significantly from 37.7 ± 20.4 % and 31.6% (range, 22.9% – 149.2%) to 28.2 ± 15.9% and 25.9% (range, 5.1% – 79.2%, p < 0.01 for each; **Table 50 & Figure 55**. In the whole period analysis again patients with low variability before conversion demonstrated a significant increase in WPV after conversion: the mean within-patient %CV of tacrolimus C_0 increased significantly from 19.3% ± 4.7% and 20.8% (range, 6.8% – 26.2%) to 26.9 % ± 9.9% and 26.6% (range, 5.8% – 57.8%, p <0.001 for each). In contrast patients with

high WPV had a significant change between the two formulations ($p < 0.01$). The WPV reduced from 39.1 ± 19.0 % and 32.2% (range, 26.5 % – 147.5%) to 31.6 ± 11.4 % and 28.2% (range, 12.8 % – 59.9%, $p < 0.01$ for each; **Table 50 & Figure 56**).

Table 50: The Individual Change of Tacrolimus WPV in High and Low Variability Patients before and after Conversion from Twice-Daily Tacrolimus to Advagraf®.

Parameter	Twice-daily tacrolimus	Advagraf®	p-value
Stable periods			
High WPV			
Mean	37.7 ± 20.4	28.2 ± 15.0	<0.01
Median (range)	31.6% (22.9% – 149.2%)	25.9% (5.1% – 79.2%	<0.01
Low WPV			
Mean	16.2 ± 4.6	21.6 ± 9.8	<0.001
Median (range)	17.4% (4.7% – 22.9%)	21.3% (4.7% – 48.6%,	<0.001
The whole period			
High WPV			
Mean	39.1 ± 19.1	31.6 ± 11.4	<0.01
Median (range)	32.2% (26.5 % – 147.5%)	28.2% (12.8 % – 59.9%)	<0.01
Low WPV			
Mean	19.3 ± 4.7	26.9 ± 9.9	<0.001
Median (range)	20.8% (6.8% – 26.2%)	26.6% (5.8% – 57.8%	<0.001

Values were compared using Kruskal-Wallis Test and ANOVA (Balanced ANOVA): Data are shown as mean ± SD and median (range).

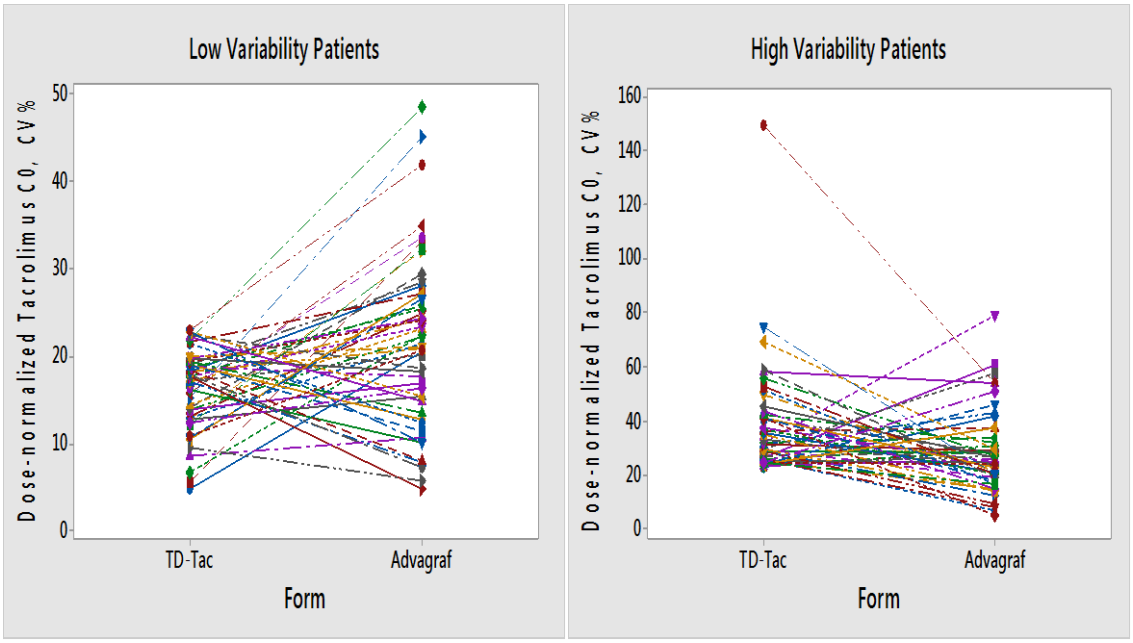


Figure 55: The Individual Change of Percent Coefficient of Variation (CV%) During Periods of Stable Tacrolimus Dose before and after Conversion from Twice-Daily Tacrolimus (TD-Tac) to Advagraf® in Patients with High and Low WPV before Conversion.

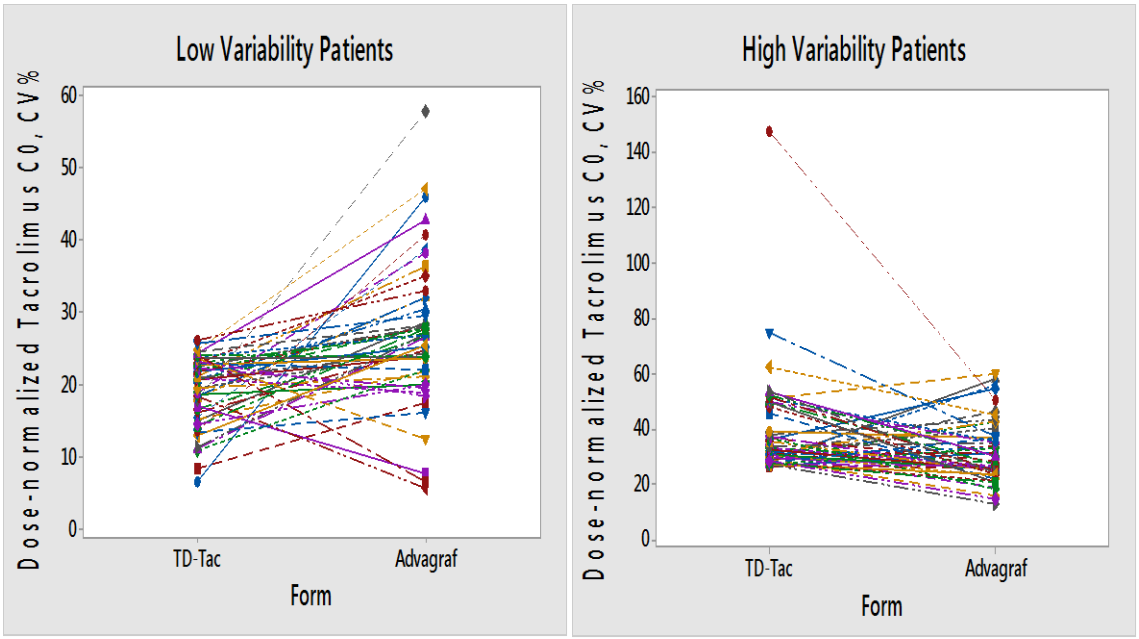


Figure 56: The Individual Change of Percent Coefficient of Variation (CV%) During the Whole Period before and after Conversion from Twice-Daily Tacrolimus (TD-Tac) to Advagraf® in Patients with High and Low WPV before Conversion.

7.3.4 Genetic Polymorphisms and WPV

*7.3.4.1 The Relationship between CYP3A5*3 Genotype and WPV*

Of the 100 patients included in our original study cohort, *CYP3A5* genotyping was available for 72 patients. For all patients, at least three C_0 results were used to calculate the within-patient variability of dose-normalized Tac C_0. Analysis of tacrolimus trough blood concentrations (C_0) was made during periods of stable tacrolimus doses and over the whole periods before and after conversion.

The wild-type genotype *CYP3A5*1/*1* was observed in 13 patients (18.1%), whereas 18 (25.0%) were heterozygous (*CYP3A5*1/*3*) and 41 (56.9%) homozygous (*CYP3A5*3/*3*) for the variant allele. The subjects were grouped according to *CYP3A5* genotype based on the presence or absence of the *CYP3A5*1* allele into 2 groups CYP3A5 expressers (n= 31); *CYP3A5*1/*1* (n = 13, male = 9: female = 4) + *CYP3A5*1/*3* (n = 18, male = 11: female = 7) and CYP3A5 non-expressers; *CYP3A5*3/*3* (n = 41, male = 25: female = 16). The baseline characteristics of these 72 patients are shown in **Table 51**. Demographic characteristics, except ethnicity were not significantly different between the genotype groups. The *CYP3A5*1* allele was more prevalent among black transplant recipients compared with that among white patients and *CYP3A5*3* allele was more prevalent in white patients than in black patients, which is in line with previous observations.

Table 51: Patient Demographics and Baseline Characteristics.

Characteristics	CYP3A5 expressers (**1/*1* plus **1/*3*) (n=31)	CYP3A5 non-expressers *(*3/*3)* (n=41)
Sex:		
Male / Female	20/11	25/16
Age	55.2 ± 11.2	55.2 ± 12.9
Body weight (kg)	73.0 ± 14.4	74.7 ± 14.8
Ethnicity:		
White	11	33
Black	12	3
Asian	8	5
Donor type:		
Living / Deceased	11/20	19/23
Primary kidney disease:		
Hypertensive nephropathy	2	3
Polycystic kidney disease	9	7
Diabetic nephropathy	4	5
IgA nephropathy	4	3
Glomerulonephritis (other)	3	7
Congenital / Reflux disease	2	2
other	2	5
Unknown	5	9

All values are shown in mean ± SD

Within-individual coefficients of variation (CV %) were calculated from analysis of variation in CYP3A5 expresser and CYP3A5 non-expresser groups were compared. Patients in each group were then divided into low and high within-individual variability subgroups using the WPV median as the cutoff value. Out the 72 patients included, 36 were considered as a low within-patient variability group, whereas 36 patients were considered as a high within-patient variability group.

During the entire pre-conversion period, the mean twice-daily tacrolimus (TD-Tac) dose was 5.6 ± 3.6 mg with dose normalized Tac C_0 of 14.5 ± 10.5 µg/L. Tacrolimus dose requirement was 1.8-fold higher in CYP3A5 expressers compared with that in CYP3A5

non-expressers (**Table 52**). The mean Tacrolimus dose and dose-normalized trough C_0 changed considerably between both genotype groups (P<0.001). Moreover, the mean of tacrolimus WPV did not differ between CYP3A5 expressers and non-expressers (28.7% ± 12.3% versus 26.3% ± 12.0%, P = 0.41, **Table 52**). Almost exactly half of CYP3A5 expresser (n = 31) and non-expressers (n = 41) exhibited low variability and the other half exhibited high variability. There was no significant difference in CV% within patients with low or high variability in each group; p= 0.26 and p= 0.43, respectively (**Table 53**).

The analysis was repeated in periods of stable tacrolimus dose. The mean of twice-daily tacrolimus dose was 5.5±3.5 mg with dose-normalized Tac C_0 of 14.4 ±10.7 µg/L. Again CYP3A5 expressers required 1.8-fold higher tacrolimus dose than CYP3A5 non-expressers. There was a significant difference in tacrolimus dose and dose-normalized trough C_0 between CYP3A5 expressers and CYP3A5 non-expressers; P<0.001, **Table 52**). The %CV of tacrolimus trough blood concentrations was significantly lower in CYP3A5 non-expressers in comparison with CYP3A5 expressers (22.4 ± 10.9% to 30.0 ± 14.5%; P =0.014). Analysis of patients with low variability demonstrated significantly lower WPV for CYP3A5 non-expressers in comparison with CYP3A5 expressers: 19.5 ± 5.9% vs. 15.5 ± 3.2 % (p < 0.05; **Table 52**). This effect was even stronger in high variability patients (41.2 ±12. 4% vs. 29.8 ±11.4%, p<0.01; **Table 53**).

During the Advagraf® post-conversion period, the results were similar to the period under twice-daily tacrolimus treatment. The mean Tacrolimus dose was 5.2 ± 3.3 mg with dose-normalized Tac C_0 of 12.9 ± 9.0 µg/L. CYP3A5 non-expressers required significantly lower Advagraf® doses (-50%, P<0.001) compared with CYP3A5 non-expressers (**Table 52**). No significant difference was observed in WPV of dose-normalized Tac C_0 between CYP3A5 expressers and CYP3A5 non-expressers (29.4% ± 11.1% versus 28.9% ± 9.6%, P = 0.82; **Table 52**). When comparing CYP3A5 expressers with non-expressers in low–variability patients no differences in the within-patient variability of dose-normalized Tac C_0 were observed (p=0.94). The same is evident for the high–variability patients (p= 0.65; **Table 53**).

The analysis was repeated in periods of stable tacrolimus dose, Advagraf® mean Tacrolimus dose was 5.1 ± 3.3 mg with dose-normalized Tac C_0 of 13.4 ± 10.2 µg/L. Again Tacrolimus dose requirement was 2.1-fold higher in CYP3A5 expressers compared with that in CYP3A5 non-expressers. A significant difference in Tacrolimus dose and dose-normalized trough C_0 was observed in both genotype groups ($p<0.001$). We observed no differences in tacrolimus WPV between CYP3A5 expressers and non-expressers in Advagraf® treatment (28.1% ± 17.4% versus 23.3% ± 9.2%, P = 0.13; **Table 52**). Only patients with high variability demonstrated a significant reduction in WPV in CYP3A5 non-expressers compared with CYP3A5 expressers: WPV decreased from 41.4 ± 15.7% to 30.3 ± 6.9% ($p < 0.01$). Additionally, no differences were observed in the within-individual variability of dose-normalized Tac C_0 between low variability patients with the *CYP3A5*1/*1* or **1/*3* versus *CYP3A5*3/*3* genotypes (P = 0.59; **Table 53**).

Conversion from TD-Tac to Advagraf® did not show any significant difference in tacrolimus dose requirement and WPV in both genotype groups either in periods of stable tacrolimus dose or periods pre-conversion or post-conversion. Additionally, WPV did not change in either CYP3A5 expressers or CYP3A5 non-expressers in both high and low variability patients after the switch.

Table 52: Within-Patient Variability in Dose-Normalized Tacrolimus Trough Concentration (Tac WPV) and *CYP3A5* Genotype.

Parameters	Twice-daily Tacrolimus		Once-daily Tacrolimus	
	*Pre-conversion period	Stable period	*Post-conversion period	Stable period
Tacrolimus dose (mg/day)				
CYP3A5 nonexpressers (**3/*3*)	4.1 ± 2.3	4.0 ± 2.2	3.5 ± 1.7	3. 4 ± 1.7
CYP3A5 expressers (**1/*1* plus **1/*3*)	7.8 ± 3.9	7.6 ± 3.9	7.5 ± 3.5	7.4 ± 3.6
	p<0.001	p<0.001	p<0.001	p<0.001
Tac C_0 (µg/L / mg/kg)				
CYP3A5 nonexpressers (**3/*3*)	18.1 ± 9.3	17.9 ± 9.6	16.4 ± 9.2	17.1 ± 10.7
CYP3A5 expressers (**1/*1* plus **1/*3*)	9.8 ± 10.3	9.8 ± 10.6	8.2 ± 6.5	8.6 ± 7.4
	p<0.001	p<0.001	p<0.001	p<0.001
Tac WPV (CV%)				
CYP3A5 nonexpressers (**3/*3*)	26.3 ± 12.0	22.4 ± 10.9	28.9 ± 9.6	23.3 ± 9.2
CYP3A5 expressers (**1/*1* plus **1/*3*)	28.7 ± 12.3	30.0 ± 14.5	29.4 ± 11.1	28.1 ± 17.4
P (**1/*1* plus **1/*3* vs. **3/*3*)	p = 0.41	p < 0.05	p = 0.82	p = 0.13

*Pre-conversion: the whole period before switching to OD-Tac where patients were on TD-Tac treatment. Post-conversion: the whole period after switching to OD-Tac where patients were on OD-Tac treatment. All values are shown in mean ± SD. Data are shown as mean ± SD. Values were compared using ANOVA (General linear model).

Table 53: High and Low Within-Patient Variability in Dose-Normalized Tacrolimus Trough Concentration and *CYP3A5*3* Genotype.

CYP3A5 Genotype	Dose-normalized Tac trough Concentration CV%	
	Low Variability (n=36)	**High Variability (n=36)**
Pre-conversion period		
CYP3A5 nonexpressers (**3/*3*)	18.0 ± 4.5	35.0 ± 11.2
CYP3A5 expressers (**1/*1* plus **1/*3*)	20.0 ± 6.1	38.0 ± 10.3
	p= 0.26	p= 0.43
Post-conversion period		
CYP3A5 nonexpressers (**3/*3*)	22.1 ± 4.8	35.9 ± 8.1
CYP3A5 expressers (**1/*1* plus **1/*3*)	22.0 ± 4.9	37.4 ± 10.3
	p=0.94	p= 0.65
TD-Tac Stable period		
CYP3A5 nonexpressers (**3/*3*)	15.5 ± 3.2	29.8 ± 11.4
CYP3A5 expressers (**1/*1* plus **1/*3*)	19. 5 ± 5.9	41.2 ± 12.4
	p < 0.05	p<0.01
OD-Tac Stable period		
CYP3A5 nonexpressers (**3/*3*)	16.7 ± 5.5	30.3 ± 6.9
CYP3A5 expressers (**1/*1* plus **1/*3*)	15.7 ± 5.6	41.4 ± 15.7
	p =0.59	p < 0.01

High variability refers to patients having high within-patient variability and Low variability refers to patients having low within-patient variability using the median as the cutoff value. All values are shown in mean ± SD. Values were compared using ANOVA (One way ANOVA).

7.4 Discussion and Conclusion

This retrospective analysis studied stable patients switched from TD-Tac to OD-Tac after kidney transplantation. There were no adverse events associated with conversion with stable graft function. The serum creatinine and estimated glomerular filtration rate did not show any significant change after conversion. Stable function after conversion has been reported by other studies (Guirado et al., 2011, Kurnatowska et al., 2011, Shuker et al., 2014). However, the study by Tinti et al. (2010) showed an improvement in the renal graft function after conversion from TD-Tac to OD-Tac. Serum creatinine and GFR showed significant improvements after conversion.

In this study, we found a significant decrease in Tac C_0 by 16% and in Tacrolimus dose-normalized C_0 by 11% after conversion and almost all the recipients required dose adjustments. This is in line with the previous observations showing that conversion from TD-Tac to OD-Tac on a 1:1 (mg: mg) basis, resulted in considerably lower Tac C_0 concentrations and sometimes required dose adjustments (Shuker et al., 2014, Tinti et al., 2010, Wehland et al., 2011). During periods of stable tacrolimus doses, the reduction was 15% in Tac C_0 and 10% in dose-normalized Tac C_0 and the majority of the recipients required dose changes. This supports our findings of the whole period analysis in which the dose-normalized Tac C_0 and dose reduced significantly after switching from TD-Tac to OD-Tac. In contrast to what would be expected from the published literature, tacrolimus C0 concentration actually increased in a significant proportion of patients leading to dose reduction on conversion from twice daily tacrolimus to Advagraf®.

Regarding WPV, our patients converted to Advagraf® did not show any significant decrease in WPV of dose-normalized Tac C_0 compared to a twice-daily tacrolimus and the same results were obtained during analysis of periods of stable tacrolimus dose. Similar observations were made in other conversion studies where they failed to show any change in Tac WPV after conversion either in small populations (van Hooff et al., 2012, Wehland et al., 2011) or large populations (Shuker et al., 2014). However, other reports demonstrate that conversion from Prograf® to Advagraf® was associated with a

significantly lower within-patient variability of tacrolimus trough concentration. A study by Alloway et al. (2005) reported a greater decrease in within patient variability of tacrolimus exposure with modified release tacrolimus versus Prograf® for African-American patients compared to Caucasian patients, although only limited data were provided. It has been reported that the conversion from twice-daily Prograf® to once-daily Advagraf® was associated with a significant decrease of within-patient variability of tacrolimus C0 (Wu et al., 2011). The discrepancy in the results may be due to differences in the study design, sample size and the inclusion and exclusion criteria of these studies. In the study by Wu et al. (2011), all patients were from a Chinese background (Taiwanese) and only patients with stable renal function and tacrolimus C_0 were included. In addition, an important limitation of their study is that the study was not randomized or double blind so patients may improve or modify their behaviour in response to the fact that they are being studied, not in response to any particular experimental manipulation.

In addition, we found that patients with low variability before conversion demonstrated a significant increase in WPV after conversion and patients with high variability before conversion associated with a significant decrease in WPV after conversion. The same results were obtained in a recent study (Shuker et al., 2014). It can be argued that the changes observed in Tac WPV after conversion is not clinically relevant. This is likely to be due to regression to the mean. From the available published data, there is certainly no clear indication that WPV is reduced on conversion from twice daily to once daily tacrolimus preparations.

In this study, we investigated the influence of *CYP3A5* genotype on tacrolimus WPV Tac C_0 in stable kidney transplant patients at least 3 months after transplantation. In this population, only 10 patients were less than 6 months post-transplantation. Our findings showed that patients treated with twice daily tacrolimus did not show any significant association between WPV of dose-normalized Tac C_0 and *CYP3A5* genotype. Moreover, no difference in CV% was observed between low or high variability patients in each group. This is in line with the previous studies investigating the impact of *CYP3A5* genotype on the Tac WPV. Pashaee et al. (2011) on mixed ethnicity patients

found no association between within-patient variability of TD-Tac clearance and *CYP3A5* genotype. A study in Korean patients demonstrated that *CYP3A5* polymorphism had no impact on TD-Tac within- patient variability (Ro et al., 2012). In both studies, they used data between 6 and 12 months post transplantation to calculate tacrolimus WPV. Additionally, these results parallel those obtained by Wu et al. (2014) who found no significant difference in TD-Tac WPV between CYP3A5 high- and low-expression.

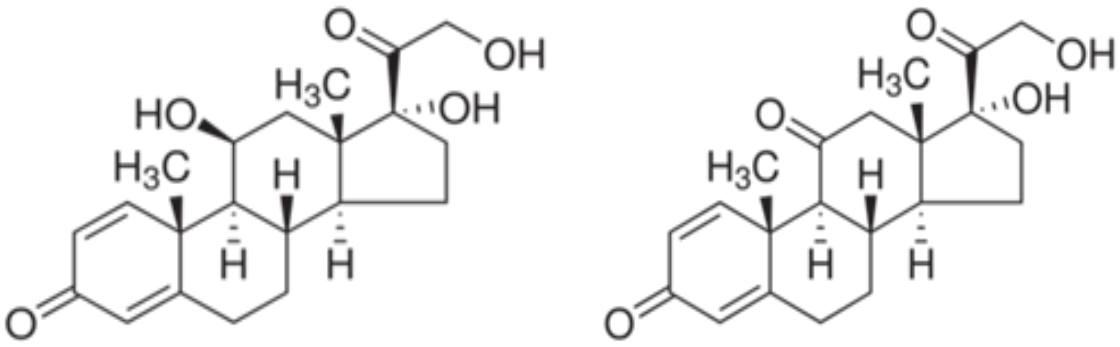
However, during periods of stable tacrolimus dose the WPV of dose-normalized Tac C_0 was significantly lower in CYP3A5 expressers compared to CYP3A5 non-expressers. Furthermore, low and high variability patients demonstrated the same significant difference in WPV for both genotype groups. Our findings are in agreement with the observations of Yong Chung et al. (2010) who reported an association between *CYP3A5* genotype and the within individual variability of tacrolimus PK parameters in healthy volunteers of two way crossover bioequivalent study. They are in contrast with the findings of Spierings et al. (2013) study on TD-Tac using 3 coinciding tacrolimus doses and showing that the within-patient variability of tacrolimus clearance was not associated with *CYP3A5* genotype in stable renal transplant recipients. This discrepancy may be due to the difference in sample size as they used only 3 tacrolimus blood concentrations and 3 coinciding tacrolimus doses and in our study at least 3 doses and 3 concentrations were used for each patient.

For once-daily tacrolimus only one study addressed the effect of *CYP3A5* genotype on Tac WPV (Wu et al., 2014). In our study, patients treated with once- daily tacrolimus, no differences in the within-patient variability of dose-normalized Tac C_0 were observed between CYP3A5 expressers and non-expressers. For high- and low-variability patients, we found no significant difference in WPV between CYP3A5 expressers and non-expressers. This is similar to results obtained from a recent study showing no significant difference in %CV of tacrolimus trough concentration between high- and low-expressers (Wu et al., 2014). During periods of stable Advagraf® dose, we observed no differences in the WPV between CYP3A5 expressers and non-expressers. We also found that only patients with high variability demonstrated a

significant reduction in WPV in CYP3A5 non-expressers compared with CYP3A5 expressers.

Conversion from TD-Tac to Advagraf® did not show any significant difference in tacrolimus WPV in both genotype groups. Additionally, WPV did not change between CYP3A5 expressers and CYP3A5 non-expressers in both high-and low-variability patients after the switch. This is partially in line with the Wu et al. (2014) study showing a specific reduction in %CV of tacrolimus trough level in patients with high-expression CYP3A5 genotype when they converted to once-daily Advagraf®. Our study has some limitations, including the retrospective nature of the study and lack of compliance data.

Chapter 8. Pharmacogenetic Associations with Prednisolone and Prednisone Exposure



Prednisolone **Prednisone**

Figure 57: Structure of Prednisolone and Prednisone.

Corticosteroids are an important component of clinical immunosuppressive therapy, and they have been used from the advent of transplantation in the 1960s. Prednisolone, a synthetic corticosteroid, is a widely used immunosuppressive agent and is commonly used to treat and prevent acute rejection after organ transplantation (**Figure 57**). Prednisone is the main metabolite of prednisolone and they are integral components of induction and maintenance immunosuppressive regimens in solid organ transplantation (Bergmann et al., 2012). Evaluating the plasma concentration of prednisolone, endogenous cortisol and their inactive metabolites, prednisone and cortisone, may help in the assessment of therapeutic response to corticosteroids or their side-effects. CYP3A and P-glycoprotein (P-gp) are involved in the metabolism of both tacrolimus and steroids. Steroids are a well-known inducer of both CYP3A and P-glycoprotein activity. It has been reported that the higher the steroid dosage used, the higher the tacrolimus dosage required to achieve target trough blood concentration (Anglicheau et al., 2003a).

Objectives of the Study

1. To develop and validate a new method for measurement of prednisolone and its metabolite prednisone in plasma using HPLC-MS/MS.
2. To explore the relationship between the *CYP3A5*3*, *ABCB1 3435*, *CYP3A4*22* and *POR*28* SNPs and prednisolone- prednisone exposure.

8.1 Validation of Liquid Chromatography – Mass Spectrometry (LC-MS/MS) Method for the Analysis of Prednisolone and Its Metabolite in Human Plasma.

8.1.1 Introduction

Several high performance liquid chromatography (HPLC) methods were reported in the published literature for determination of prednisolone and prednisone in plasma. The disadvantage of these HPLC methods is that they consume large volumes of plasma (Gai et al., 2005, Cheng et al., 1988) and are less sensitive (Gai et al., 2005, Huber et al., 1990, Alvinerie et al., 1990) and have long run times (Cheng et al., 1988, Lasic et al., 1989). Ionita and Akhlaghi (2010) described an LC-MS/MS method that achieved a good sensitivity for determination of unbound prednisolone and prednisone. The samples were separated by the ultrafiltration of plasma using Microcon centrifugal filter devices. Methlie et al. (2013) developed an LC–MS/MS method that quantifies endogenous and synthetic corticosteroid as prednisolone and prednisone in serum using long extraction method. Evaluating the plasma concentration of prednisolone, endogenous cortisol and their inactive metabolites, prednisone and cortisone, may help in the assessment of therapeutic response to corticosteroids or their side-effects.

8.1.2 Materials and Methods

8.1.2.1 Prednisolone-Prednisone Analysis

Prednisolone and prednisone plasma concentration were measured using validated HPLC based assay with tandem mass spectrometry detection. The analytical method was based on using the following

8.1.2.1.1 Instrumentation

Solvent delivery was achieved using an Agilent series 1100 pump. Sample injection was performed by using an Agilent series 1100 auto injector. The column temperature was adjusted using an Agilent series 1100 column oven. Detection was by an API4000 Mass spectrometer (AB Sciex, England). A Windows PC computer running Analyst 1.3.2

software was used to control the HPLC/MS, record the output from the detector, perform integration of peak areas and calculate the prednisolone and prednisone concentrations. The Analyst software was supplied by Applied Biosystems, England. The NM20ZA high purity nitrogen and air generators were supplied by Peak Scientific Instruments, Scotland.

8.1.2.1.2 Chemicals & Reagents

Prednisolone, prednisone, cortisol, cortisone, dexamethasone (internal standard) and orange G were supplied by Sigma-Aldrich. Plasma samples containing EDTA anticoagulant were obtained from Biological Specialty Corp. HPLC-graded ethyl acetate and acetic acid (100%) were purchased from VWR together with sodium hydroxide. HPLC-graded methyl-tert-butyl ether and methanol (HPLC grade) were obtained from Rathburn Chemicals Ltd. Sodium chloride was supplied by BDH Prolabo. De-ionised water was prepared on site using Millipore (water purification system).

8.1.2.1.3 Stock Solutions

Stock solutions of prednisolone (1 mg/L) and prednisone (200.0µg/mL) were prepared in methanol and a combined sub-stock of prednisolone and prednisone stocks was prepared by further dilution in drug-free human plasma to obtain calibration and quality control (QC) stocks.

Calibration standards were spiked with prednisolone-prednisone calibration stock solution to give final concentrations of 2.5, 5.0, 12.5, 50.0, 125.0, 250.0 and 375.0 µg/L for prednisolone and 0.5, 1.0, 2.5, 10.0, 25.0, 50.0 and 75.0 µg/L for prednisone. Aliquots of these standard solutions were then stored at -20°C until analysis. Three QC samples were prepared by using the same procedure at concentrations of 25.0, 125.0 and 250.0 µg/L for prednisolone and 5.0, 25.0 and 50.0 µg/L for prednisone in human plasma. Dexamethasone stock solutions (1000 mg/L) was prepared in methanol and the internal standard stock solution of dexamethasone was prepared by further dilution in deionized water at a concentration of 1 mg/L and stored at approximately 4ºC.

8.1.2.1.4 Extraction Procedure:

Calibrators / Controls / Patients samples (100µL), internal standard solution (50µL), alkaline sodium chloride solution (250µL) and 1mL extracting solvent (ethyl acetate: methyl-tert-butyl ether 1:1) were pipetted into pre-labelled 2mL polypropylene tubes. The alkaline sodium chloride solution consists of sodium chloride (23g), 1ml of sodium hydroxide solution 40% (w/w), orange G (10 mg) and de-ionized water (100ml) The contents of the tubes were mixed for 5 min and then centrifuged at; 13,200 rpm for 2.5 min using Eppendorf 5415D Centrifuge (Eppendorf, England, UK). The entire top solvent layer was then transferred to a 4.5 mL conical polypropylene tube and transferred to the SpeedVac and evaporated to dryness at high heat setting. The dried extracts were reconstituted with 1 mL of 25% methanol, vortex mixed for 20 seconds and then centrifuged at approximately 3500 RPM for approximately 2 minutes using Heraeus Megafuge 11 Centrifuge (Thermo Scientific, England, UK). The extract was then transferred to labelled auto-sampler tubes and a 10µL aliquot of each extract was injected onto the column (**Figure 58**).

8.1.2.1.5 Chromatographic and Mass Spectrometric Conditions

LC-MS/MS method was validated for the analysis of prednisolone and its metabolite prednisone in plasma. The separation was performed using an analytical Hypercarb 30mm x 3mm, 5µm column (Thermo Scientific, UK) at temperature of 50°C. The mobile phase was pumped isocratically at a flow rate of 0.6 mL/min and consisted of 0.1% acetic acid in methanol. The sample injection volume was 10 µL. Tandem mass spectrometric detection and quantification was performed in the negative electrospray ionization mode using multiple-reaction monitoring (MRM) mode. The transition selected were m/z 419.3/329 for prednisolone, 417.13 /327 for prednisone and m/z 451.2/361.1 for dexamethasone (**Figure 58**). MS Settings: An API4000 triple quadruple mass spectrometer equipped with a turbo-ion spray (heated electro-spray, ESI) was used to introduce the sample into the mass spectrometer. Nitrogen was used as the collision gas. Gas settings in ml/min were: Collision gas: 4, curtain gas: 10, ion source gas 1: 40, ion source gas 2: 40. Ion spray voltage: 4200 V, and temperature: 500°C. Entrance

potential (EP) and collision energy (CE) in voltage were 10 and 22 V, respectively for all compounds and the dwell times were set at 300 ms. Declustering Potential (DP) was 50 for both prednisolone and prednisone and 20 for dexamethasone. The collision cell exit potential (CXP) in voltage was 7, 9 and 15 V, for prednisolone, prednisone and dexamethasone, respectively.

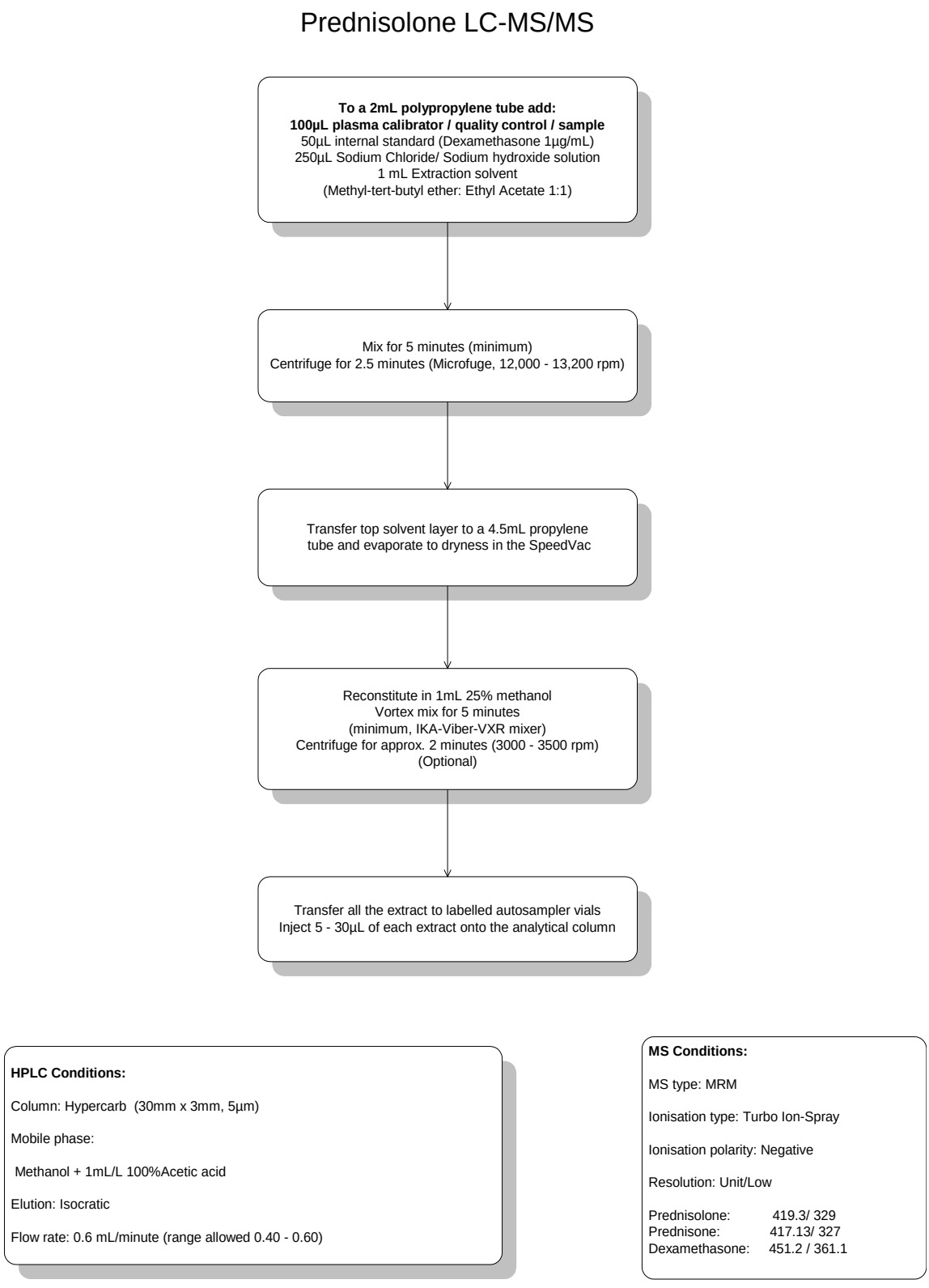


Figure 58: Schematic Diagram of the Prednisolone and Prednisone Extraction Procedure.

8.1.2.2 Method Development and Validation

An analytical method for determination of prednisolone and prednisone plasma concentration was developed and validated according to FDA guidelines. The method included determination of accuracy, precision, selectivity, sensitivity, reproducibility, and stability (FDA., 2001).

***8.1.2.2.1** Specificity*

The specificity and selectivity of the assay were investigated by comparing the retention times of prednisolone and prednisone detected in an assay of six drug-free and four prednisolone and prednisone spiked plasma samples. These samples were extracted using the above mentioned extraction method. The signal to noise ratio should be more than 5.

8.1.2.2.2 Matrix Effect

The matrix effect of the method was evaluated by analysis of 6 different batches of human plasma. Matrix effects were assessed by comparison of peak area measurements obtained from human plasma extracts spiked with prednisolone and prednisone at the QC2 level after extraction with those obtained from aqueous solutions.

8.1.2.2.3 Calibration/Linearity

Linearity was assessed by analysis of 7 non-zero calibrators of prednisolone and prednisone calibration curve in duplicate over a range 2.5 – 375.0 µg/L for prednisolone and 0.5 – 75.0 µg/L for prednisone with the internal standard (Dexamethasone). The concentrations of these calibrators were 2.5, 5.0, 12.5, 50.0, 125.0, 250.0 and 375.0 µg/L for prednisolone and 0.5, 1.0, 2.5, 10.0, 25.0, 50.0 and 75.0 µg/L for prednisone. The calibration curve also consisted of a blank sample (plasma sample analysed with internal standard), double blank sample (plasma sample analysed without internal standard). The correlation coefficient (r) between concentration and peak area ratio should be equivalent to, or better than 0.98.

The following conditions were met in developing a calibration curve for the back calculated concentrations for calibration standards:

• No more than ±20% deviation of the lower limit of quantitation (LLOQ) from actual concentration

• No more than ±15% deviation of calibrators other than LLOQ from actual concentration.

At least 75% of the non-zero standards should meet the above criteria, including the LLOQ and the calibration standard at the highest concentration, upper limit of quantitation (ULOQ). Excluding the standards should not change the regression model used.

8.1.2.2.4 Accuracy and Precision

Accuracy was determined by analysis of low, medium and high quality control samples. The low, medium and high quality control values were 25.0, 125.0 and 250.0 µg/L, respectively, for prednisolone and 5.0, 25.0 and 50.0 µg/L, respectively, for prednisone. Accuracy should be measured using a minimum of five determinations per concentration. The mean values should be within 15% of the actual value.

The precision or repeatability were measured both within-batch and between-batch by the analysis of three quality control (QC) samples. Within-run, intra-batch precision assesses precision during a single analytical run. While between-run, inter-batch precision measures precision with time, and may involve different analysts, equipment, reagents, and laboratories. Precision should be measured using a minimum of five determinations per concentration so the QCs were assayed six times in five separate essays on separate days. The precision determined at each concentration level should not exceed 15% of the coefficient of variation (CV), the ratio of the standard deviation to the mean. For calculating accuracy and precision the following formulae were used:

Accuracy

$$\text{Acc [\%]} = 100 \text{ x } \frac{\text{Mean Measured Concentration}}{\text{Nominal Concentration}}$$

Precision

$$\text{CV[\%]} = 100 \text{ x } \frac{\text{Standard Deviation}}{\text{Mean Measured Concentration}}$$

8.1.2.2.5 Recovery

Absolute recovery of prednisolone and prednisone was performed by comparing the analytical results for the extracted samples, using human plasma spiked with three different concentrations (Quality control concentrations), with non-extracted standards that represent 100% recovery. Recovery was tested in quadruplicate with the same batch of human plasma used to prepare the quality control samples. Absolute recovery of dexamethasone was tested at a nominal concentration of 50 µg/L. Mean and standard deviation were calculated from at least three measurements at each level. The absolute recovery relates to the extraction efficiency of the analytical method and it does not need to be 100%, but it should be consistent, precise, and reproducible. The extraction recovery of the test compound was calculated as follows:

$$\text{Recovery [\%]} = \frac{\text{Mean extract ratio}}{\text{Mean reference ratio}} \text{ x } 100$$

8.1.2.2.6 Stability

For stability determination samples were prepared from freshly prepared stock solutions. Stability evaluation includes the stability during sample collection and handling, after short-term (bench top, room temperature) storage, and after going through freeze and thaw cycles and the analytical process. Conditions used in stability experiments reflect situations likely to be encountered during actual sample handling

and analysis. The procedure also includes an evaluation of analyte stability in stock solution.

8.1.2.2.6.1 Short term temperature stability

The stability of three quality control samples was examined at ambient temperature, approximately 20ºC and at approximately 4ºC for a period of 24 hours. Four measurements were made at each concentration of these QC samples.

Sets of stability samples subjected to 3 freeze and thaw cycles at nominal –20°C were determined against freshly prepared calibration standards. Four measurements were made at each concentration of these QC samples.

8.1.2.2.6.2 Stability of Extracted Samples

The stability of processed samples' extracts, including the resident time in the auto-sampler, was measured in quality control samples. These quality control samples were kept in the auto-sampler at room temperature. The run was injected at t_0 and approximately 16 h later. Each sample was quantified with the calibration curve injected at the same time. For each level, the mean value, the standard deviation, the precision and the accuracy were calculated at t_0 and t_{16} h.

Acceptance criteria:

At t_0 and t_{16} h, the precision should be equal to or less than 15% and the accuracy should be between 85% and 115% of the nominal concentration for each level to demonstrate the stability of the compound. Moreover, for each level, at least 4 replicates out of 6 should have a relative error within ±15%.

8.1.2.2.7 Batch analysis and data reporting

Each analytical batch contained seven, non-zero, calibrators, assayed in duplicate. The three quality control samples, assayed in duplicate, were dispersed throughout the analytical batch. A minimum of two sets of quality control samples at each concentration was included in each full analytical batch.

The calibration curves and calculation of prednisolone and prednisone concentrations used $1/x^2$ weighted least-squares regression to describe the concentration-response relationship. The peak area ratio, correlation coefficient (r) and the slope of the calibration line were calculated using the peak area data by the Analyst 1.3.2 software.

Assay Acceptance Criteria

For study sample results to be accepted the following acceptance criteria must be met for the analytical batch. In the case of a rejected analytical batch, all study samples of that analytical batch should be re-analysed.

A. Calibration Curve

The correlation coefficient (r) between concentration and peak area ratio should be equivalent to, or better than, 0.98.

B. Calibration Standards

The following conditions should be met in developing a calibration curve for the back calculated concentrations for calibration standards:

- no more than ±20% deviation of the LLOQ from actual concentration
- no more than ±15% deviation of calibrators other than LLOQ from actual concentration

At least 75% of the non-zero standards should meet the above criteria, including the LLOQ and the calibration standard at the highest concentration. Excluding the standards should not change the regression model used.

C. Quality Control Samples

The concentrations of both the quality control samples and the patient samples were calculated by using the peak areas of each analyte with respect to the peak areas of the appropriate internal standard.

The following conditions should be met for batch acceptance:

- ❖ no more than ±15% deviation of quality controls from their actual concentration

At least 67% of the controls must meet the above criteria.

8.1.3 Results

Optimization of detector parameters for tandem mass spectrometric detection of the analytes (prednisolone and prednisone) was determined through direct infusion of each analyte or internal standard (Dexamethasone) at a flow rate of 0.6 mL/min. Each of the drugs was dissolved in 10% acetic acid: methanol (50:50) with a concentration of 1µg/mL.

The molecular structure and molecular weight of prednisolone, prednisone, dexamethasone and endogenous steroids (Cortisone and Cortisol) is shown in **Figure 59**. Parent ion scan (Q1 scan) and product ion scan (Q3). The fragment ions selected for the assay are shown in **Table 54**. Endogenous steroids (Cortisone and Cortisol) were tested to make sure there is no interfering peak at the retention times for prednisone and prednisolone (**Figure 60**).

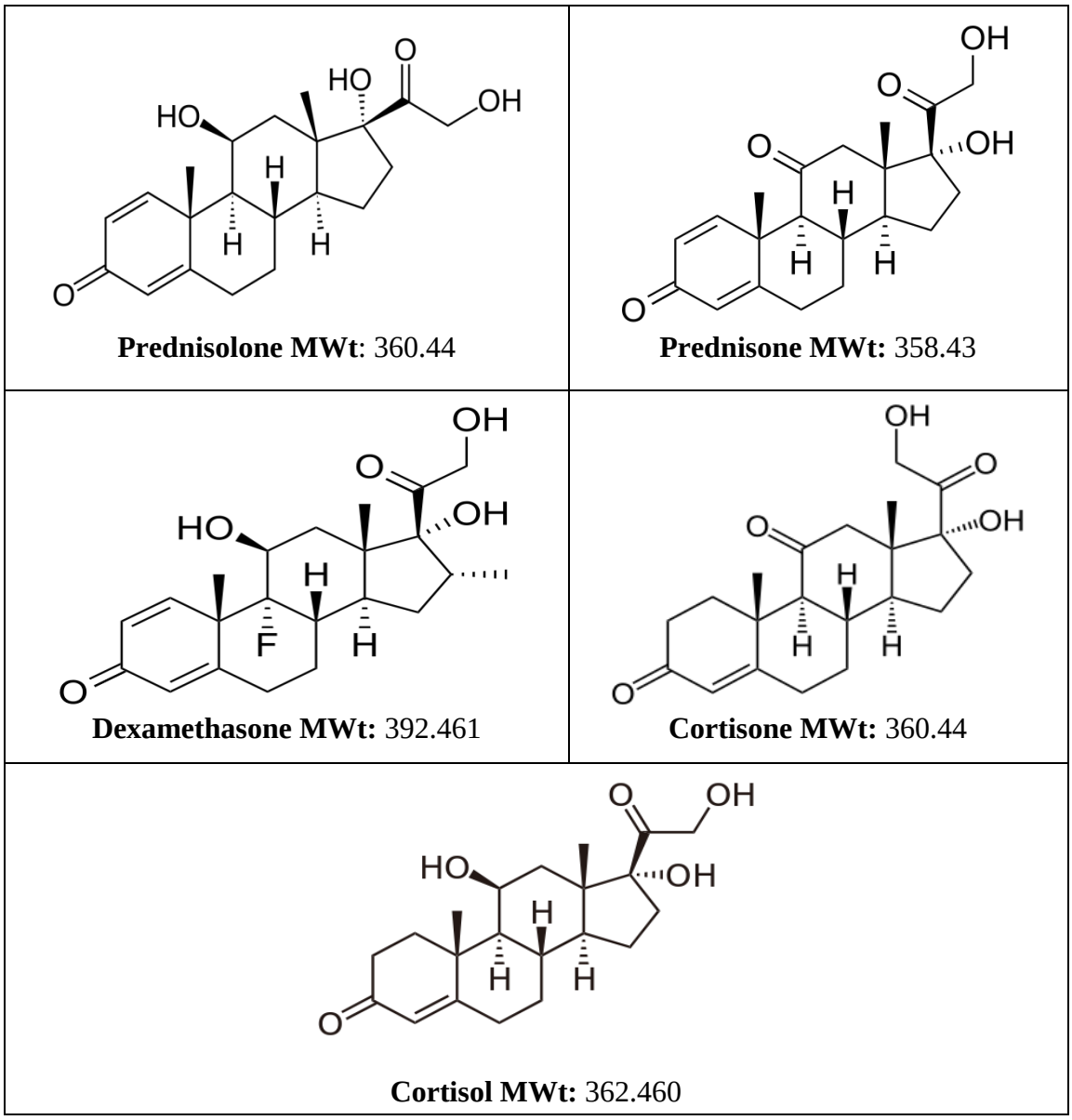


Figure 59: Molecular Structure and Molecular Weight of Separated Steroids.

Table 54: Analyte Mass Transitions

Analyte Name	Prednisolone	Prednisone	Dexamethasone	Cortisone	Cortisol
Initial ion (m/z) Q1 Mass (amu)	419.25	417.25	451.22	419.3	421.1
Product ion (m/z) Q3 Mass (amu)	329.00	327.00	361.10	329.0	331.0
Ionisation mode	Negative	Negative	Negative	Negative	Negative

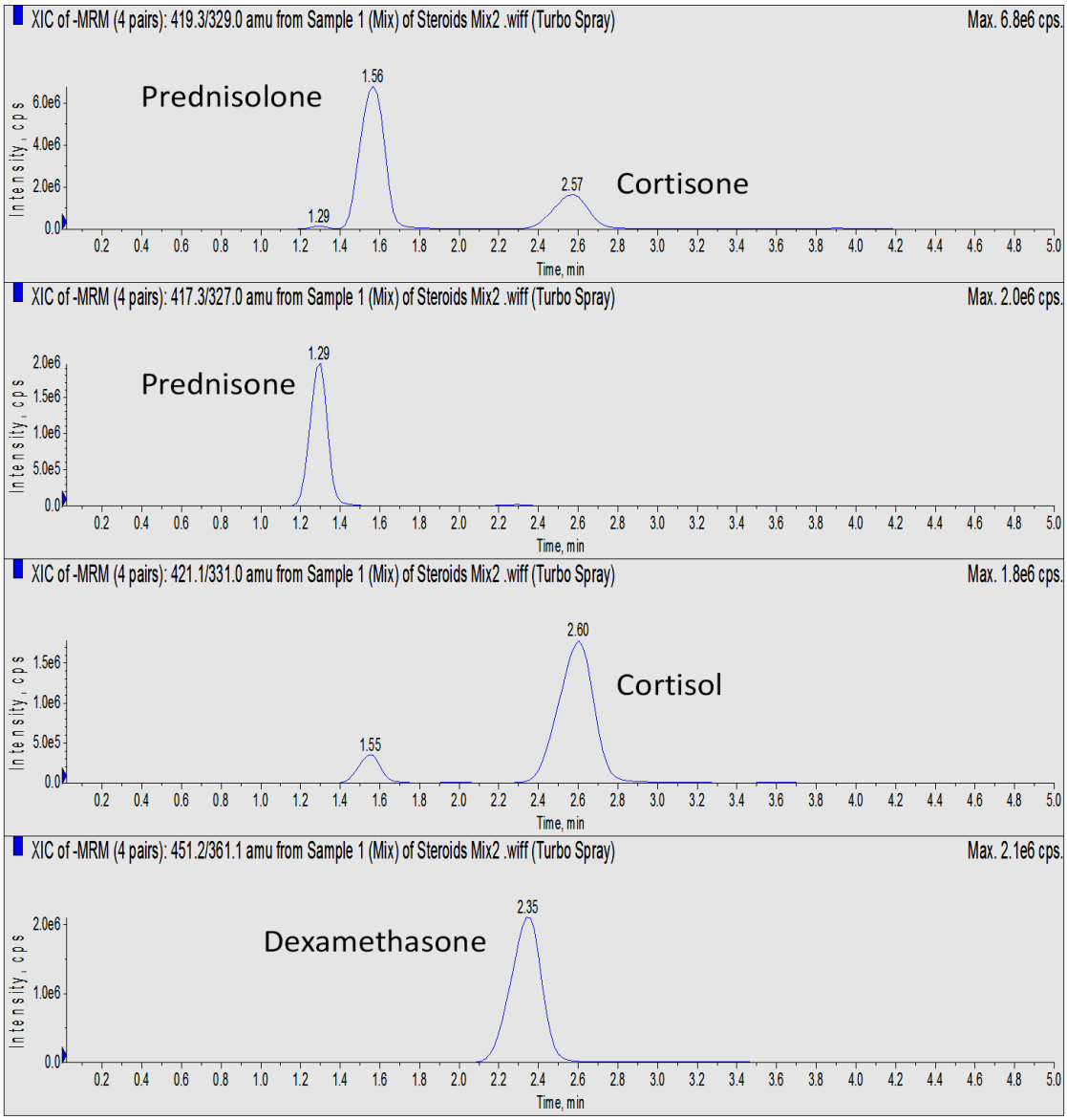


Figure 60: Chromatogram Displayed Separation of Steroids.

8.1.3.1 Specificity

No significant interfering peaks were found at the retention time of prednisolone, prednisone and the internal standard as shown in **Figure 61**& **Figure 62.** The peak to noise ratio was greater than 5 for all of them. **Figure 61** shows the chromatograms obtained from blank plasma, plasma spiked with 2.5 µg/L and 375 µg/L for prednisolone and 0.5 µg/L and 75 µg/L for prednisone. **Figure 62** shows the chromatograms of blank plasma and 50 µg/L dexamethasone spiked plasma.

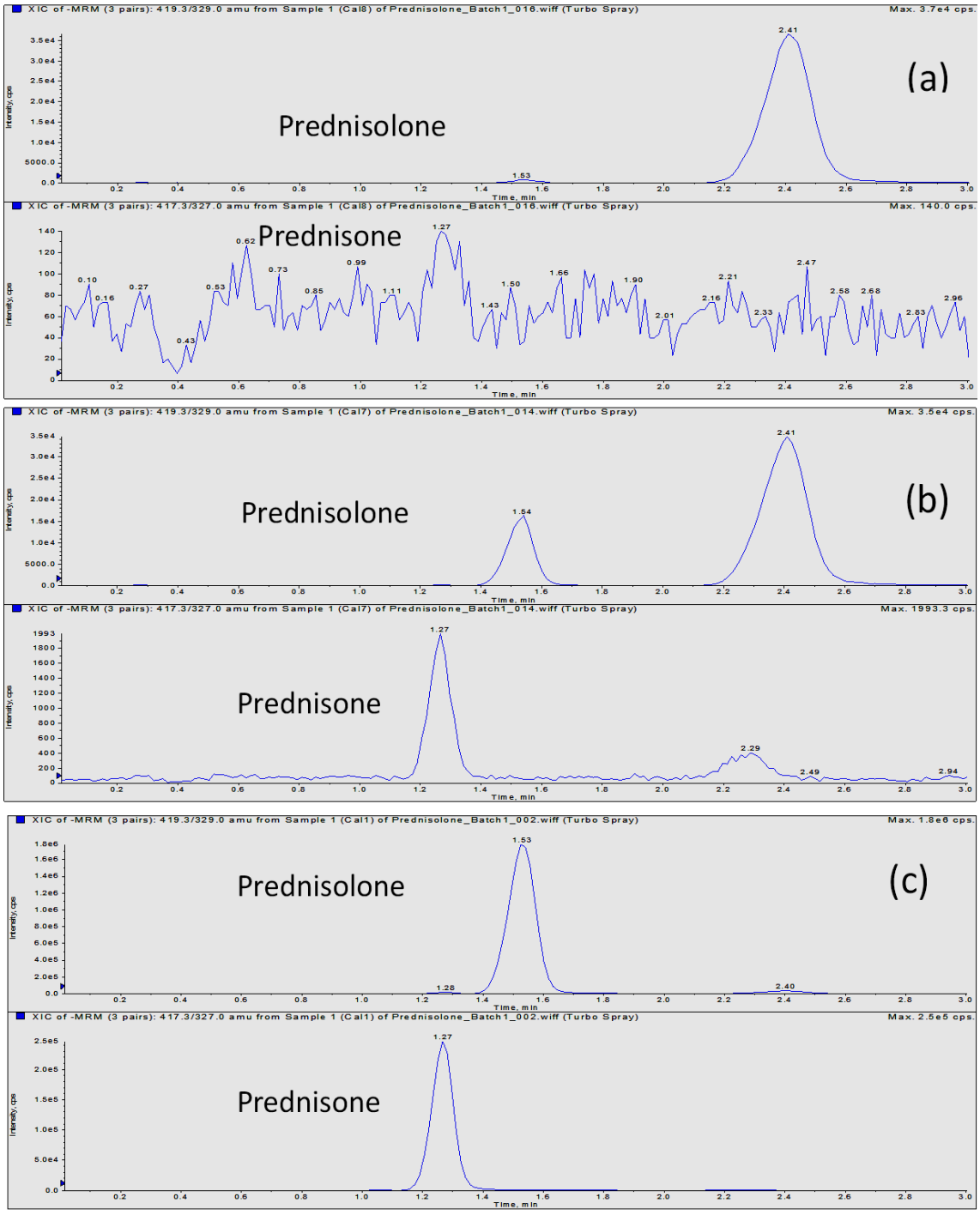


Figure 61: Chromatograms Obtained from Extracted Blank Plasma Free from Prednisolone and Prednisone (a), Blank Plasma Spiked with 2.5 µg/L Prednisolone and 0.5 µg/L Prednisone (b) and Blank Plasma Spiked with 375 µg/L Prednisolone and 75 µg/L Prednisone (c).

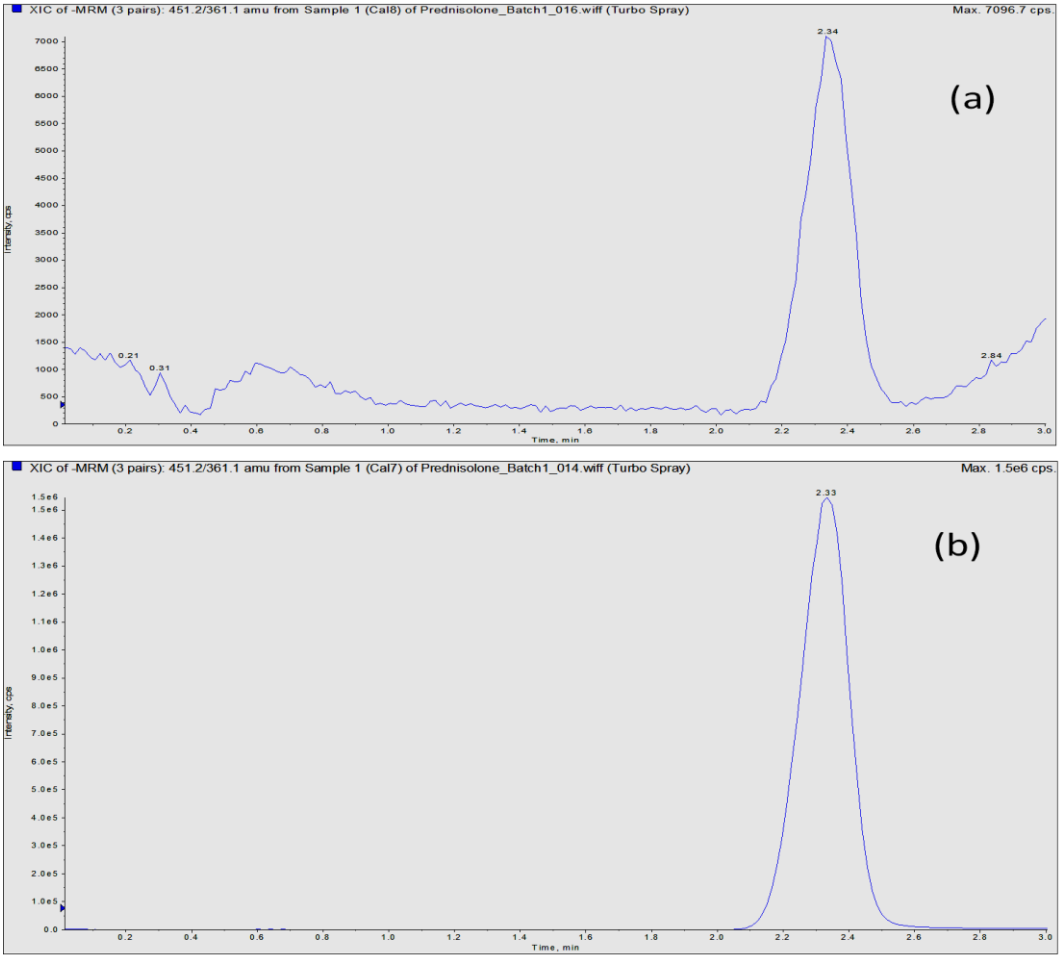


Figure 62: Chromatograms Obtained from Extracted Blank Plasma (a) and Blank Plasma Spiked with 50 µg/L Dexamethasone (b).

8.1.3.2 Matrix Effect

Prednisolone and prednisone were tested at the same nominal concentration as the middle quality control sample, with six different plasma samples. Peak area measurements obtained from post extracted plasma spiked with prednisolone and prednisone and the internal standard at the same concentrations as the middle quality control sample were compared to the peak area measurements obtained from the spiking solutions. From the obtained results, the matrix effects were considered acceptable (**Table 55**).

Table 55: Matrix Effect for Prednisolone, Prednisone and the Internal Standard.

Prednisolone

	Data	**100% QC2**	**Sample**	**Error**
Matrix.	N =	4 of 4	6 of 6	
	Data Point #1	7777956	7702121	-0.9%
	Data Point #2	7606603	7545712	-2.9%
	Data Point #3	7788381	7691022	-1.0%
	Data Point #4	7905249	7327626	-5.7%
	Data Point #5		7606565	-2.1%
	Data Point #6		7579525	-2.5%
	Mean	7769547	7575429	-2.5%

Prednisone

	Data	**100% QC2**	**Sample**	**Error**
Matrix.	N =	4 of 4	6 of 6	
	Data Point #1	1046568	1052493	-0.6%
	Data Point #2	1038420	1045274	-1.3%
	Data Point #3	1075698	1050858	-0.8%
	Data Point #4	1075516	1063013	0.4%
	Data Point #5		1055722	-0.3%
	Data Point #6		1048737	-1.0%
	Mean	1059051	1052683	-0.6%

Dexamethasone

	Data	**100% QC2**	**Sample**	**Error**
Matrix.	N =	4 of 4	6 of 6	
	Data Point #1	35500987	34880804	-0.9%
	Data Point #2	34515090	35085701	-0.3%
	Data Point #3	35363333	35125057	-0.2%
	Data Point #4	35361783	33931612	-3.6%
	Data Point #5		34733210	-1.3%
	Data Point #6		35297598	0.3%
	Mean	35185298	34842330	-1.0%

All values show the peak area for each analyte

8.1.3.3 Calibration

The calibration curves contained seven, non-zero, calibrators, containing both prednisolone and prednisone, assayed in duplicate. Nominal values for prednisolone were 2.5, 5.0, 12.5, 50.0, 125.0, 250.0 and 375.0 µg/L. Nominal values for prednisone were 0.5, 1.0, 2.5, 10.0, 25.0, 50.0 and 75.0 µg/L. Two prednisolone and prednisone-free samples were analysed, one with the internal standard and one without the internal standard; neither were included when fitting the calibration line.

The peak area ratios, regression coefficients and the slopes of the calibration lines, etc. were calculated from the peak area data by the Analyst program. The correlation coefficient (r) between concentration and peak area ratio is ≥ 0.9976 for all curves. This meets the acceptance criteria that the correlation coefficient (r) should be equivalent to, or better than, 0.98. **Table 56** & **Table 57** summarised the mean of the results obtained from five curves.

Table 56: Prednisolone Calibration Curve Parameters

Analytical run	a0	a1	a2	Correlation coefficient (r)
Batch1	0.000233	0.00237	-0.000000967	0.9998
Batch2	0.001510	0.00235	-0.000000929	0.9997
Batch3	0.000448	0.00249	-0.000001150	0.9997
Batch4	0.000508	0.00245	-0.000001120	0.9997
Batch5	0.000746	0.00234	-0.000000973	0.9992

$y=a2x^2+a1x+a0$

Table 57: Prednisone Calibration Curve Parameters

Analytical run	Slope	Intercept	Correlation coefficient (r)
Batch1	0.0000335	0.00116	0.9989
Batch2	0.0001740	0.00108	0.9993
Batch3	0.0000765	0.00106	0.9993
Batch4	0.0000596	0.00107	0.9997
Batch5	0.0001470	0.00117	0.9976

y= a1x+a0

8.1.3.4 Accuracy and Precision

The three quality control samples were, initially, each extracted six times in three batches. Subsequently, the three quality control samples were each extracted six times in two additional batches. On each occasion a separate calibration curve was extracted.

The criteria for the acceptance of the quality control samples were that both the precision and the mean measured concentration should be within ±15% of the expected value. Using these criteria, accuracy of the quality control samples for both prednisolone and prednisone were well within the ranges allowed (**Table 58 & Table 59**).

Table 58: Prednisolone Within and Between-Assay Repeatability.

		QC1	QC2	QC3
Batch1	**Expected Conc.(µg/L)**	25.0	125.0	250.0
	N =	6 of 6	6 of 6	6 of 6
	Data Point #1	24.8	110.1	260.1
	Data Point #2	26.3	135.2	252.5
	Data Point #3	25.1	140.8	232.5
	Data Point #4	23.6	138.1	255.0
	Data Point #5	26.0	132.1	258.3
	Data Point #6	25.8	135.4	250.0
	Mean	25.3	132.0	251.4
	Standard Dev.	1.0	11.1	9.9
	%CV	3.9	8.4	4.0
	Accuracy	101.0	105.6	100.6
Batch2	**Expected Conc.(µg/L)**	**25.0**	**125.0**	**250.0**
	N =	6 of 6	6 of 6	6 of 6
	Data Point #1	25.9	139.2	258.0
	Data Point #2	25.3	125.5	251.2
	Data Point #3	25.0	128.4	253.2
	Data Point #4	26.6	132.9	255.6
	Data Point #5	25.9	130.4	258.5
	Data Point #6	25.2	119.3	254.6
	Mean	25.6	129.3	255.2
	Standard Dev.	0.6	6.7	2.8
	%CV	2.2	5.2	1.1
	Accuracy	102.6	103.4	102.1
Batch3	**Expected Conc.(µg/L)**	**25.0**	**125.0**	**250.0**
	N =	6 of 6	6 of 6	6 of 6
	Data Point #1	26.7	139.9	248.2
	Data Point #2	26.5	138.6	248.8
	Data Point #3	25.6	138.9	246.0
	Data Point #4	27.2	125.3	243.0
	Data Point #5	26.5	122.8	244.3
	Data Point #6	27.1	119.7	239.5
	Mean	26.6	130.9	245.0
	Standard Dev.	0.6	9.3	3.5
	%CV	2.2	7.1	1.4
	Accuracy	106.4	104.7	98.0
Batch4	**Expected Conc.(µg/L)**	**25.0**	**125.0**	**250.0**
	N =	6 of 6	6 of 6	6 of 6
	Data Point #1	27.1	139.0	247.6

	Data Point #2	26.0	136.3	240.9
	Data Point #3	25.3	137.3	243.1
	Data Point #4	26.8	126.7	242.1
	Data Point #5	26.8	123.7	249.1
	Data Point #6	26.6	121.2	245.1
	Mean	26.4	130.7	244.7
	Standard Dev.	0.7	7.7	3.2
	%CV	2.5	5.9	1.3
	Accuracy	105.7	104.5	97.9
Batch5	**Expected Conc.(µg/L)**	**25.0**	**125.0**	**250.0**
	N =	6 of 6	6 of 6	6 of 6
	Data Point #1	24.5	121.3	247.3
	Data Point #2	24.9	117.6	233.0
	Data Point #3	23.1	114.4	232.3
	Data Point #4	25.5	123.5	260.0
	Data Point #5	25.0	121.6	241.9
	Data Point #6	24.9	118.0	239.6
	Mean	24.6	119.4	242.4
	Standard Dev.	0.8	3.4	10.3
	%CV	3.4	2.8	4.3
	Accuracy	98.6	95.5	96.9

Within-assay repeatability

	QC1	QC2	QC3
Expected Conc.(µg/L)	**25.0**	**125.0**	**250.0**
N =	30 of 30	30 of 30	30 of 30
Mean	25.7	128.4	247.7
Standard Dev.	1.0	8.8	8.0
%CV	4.0	6.9	3.2
Accuracy	102.9	102.8	99.1

Between-assay repeatability

	QC1	QC2	QC3
Expected Conc.(µg/L)	**25.0**	**125.0**	**250.0**
N =	5 of 5	5 of 5	5 of 5
Mean	25.7	128.4	247.7
Standard Dev.	0.8	5.1	5.4
%CV	3.2	4.0	2.2
Accuracy	102.9	102.8	99.1

Table 59: Prednisone Within and Between-Assay Repeatability.

	Data	QC1	QC2	QC3
Batch1	**Expected Conc.(µg/L)**	**5.0**	**25.0**	**50.0**
	N=	6 of 6	6 of 6	6 of 6
	Data Point #1	5.1	21.3	50.3
	Data Point #2	5.3	26.1	48.1
	Data Point #3	5.2	27.8	45.2
	Data Point #4	4.9	27.2	47.7
	Data Point #5	5.2	26.5	49.2
	Data Point #6	5.1	27.3	47.8
	Mean	5.1	26.0	48.0
	Standard Dev.	0.1	2.4	1.7
	%CV	2.7	9.3	3.5
	%Accuracy	102.5	104.1	96.1
Batch2	**Expected Conc.(µg/L)**	**5.0**	**25.0**	**50.0**
	N =	6 of 6	6 of 6	6 of 6
	Data Point #1	5.3	27.6	49.8
	Data Point #2	5.2	24.6	48.9
	Data Point #3	4.9	26.3	49.0
	Data Point #4	5.6	26.5	46.6
	Data Point #5	5.4	26.3	49.4
	Data Point #6	5.1	23.5	49.2
	Mean	5.2	25.8	48.8
	Standard Dev.	0.2	1.5	1.1
	%CV	4.3	5.7	2.3
	Accuracy	104.7	103.3	97.6
Batch3	**Expected Conc.(µg/L)**	**5.0**	**25.0**	**50.0**
	N =	6 of 6	6 of 6	6 of 6
	Data Point #1	5.5	27.4	46.1
	Data Point #2	5.3	26.8	46.0
	Data Point #3	5.1	26.5	43.9
	Data Point #4	5.2	24.1	46.5
	Data Point #5	5.4	22.9	44.5
	Data Point #6	5.2	22.7	43.4
	Mean	5.3	25.1	45.1
	Standard Dev.	0.1	2.1	1.3
	%CV	2.3	8.3	2.9
	Accuracy	105.5	100.2	90.1
Batch4	**Expected Conc.(µg/L)**	**5.0**	**25.0**	**50.0**
	N =	6 of 6	6 of 6	6 of 6
	Data Point #1	5.4	27.3	46.4

	Data Point #2	5.1	26.7	45.1
	Data Point #3	5.1	26.2	43.3
	Data Point #4	5.0	24.3	45.9
	Data Point #5	5.4	23.4	45.5
	Data Point #6	5.2	23.5	43.5
	Mean	5.2	25.2	44.9
	Standard Dev.	0.2	1.7	1.3
	%CV	3.4	6.8	2.9
	Accuracy	104.4	100.9	89.9
Batch5	**Expected Conc.(µg/L)**	**5.0**	**25.0**	**50.0**
	N =	6 of 6	6 of 6	6 of 6
	Data Point #1	5.7	23.0	47.7
	Data Point #2	5.2	24.7	43.7
	Data Point #3	4.5	23.9	44.4
	Data Point #4	4.8	23.3	53.8
	Data Point #5	4.9	25.5	52.8
	Data Point #6	4.7	24.7	45.1
	Mean	5.0	24.2	47.9
	Standard Dev.	0.4	0.9	4.4
	%CV	8.4	3.9	9.2
	Accuracy	99.3	96.8	95.8

Within-assay repeatability

	QC1	**QC2**	**QC3**
Expected Conc.(µg/L)	**5.0**	**25.0**	**50.0**
N =	30 of 30	30 of 30	30 of 30
Mean	5.2	25.3	47.0
Standard Dev.	0.3	1.8	2.7
%CV	4.9	7.1	5.8
Accuracy	103.3	101.1	93.9

Between-assay repeatability

	QC1	**QC2**	**QC3**
Expected Conc.(µg/L)	**5.0**	**25.0**	**50.0**
N =	5 of 5	5 of 5	5 of 5
Mean	5.2	25.3	47.0
Standard Dev.	0.1	0.7	1.8
%CV	2.4	2.9	3.9
Accuracy	103.3	101.1	93.9

8.1.3.5 Recovery

Recovery was assessed by comparison of peak area measurements obtained from human plasma spiked prior to extraction with those obtained from human plasma extracts spiked after extraction. The recovery percentage was 77.7% to 79.2% for prednisolone and 72.9% to 73.5% for prednisone (**Table 60**). The extraction recovery for dexamethasone was 82.1%. The recovery for all three analytes was acceptable.

Table 60: Prednisolone, Prednisone and Internal Standard Relative Recovery.

Prednisolone

	QC1 100%	**QC1 Ext**	**Recovery**
N =	4 of 4	4 of 4	
Data Point #1	1908337	1465785	76.8%
Data Point #2	1819192	1440493	79.2%
Data Point #3	1816171	1410251	77.7%
Data Point #4	1805942	1412439	78.2%
Mean	1837411	1432242	78.0%
	QC2 100%	**QC2 Ext**	**Recovery**
N =	4 of 4	4 of 4	
Data Point #1	8157898	6290393	77.1%
Data Point #2	8054867	6494499	80.6%
Data Point #3	8111873	6089051	75.1%
Data Point #4	8163250	6355247	77.9%
Mean	8121972	6307298	77.7%
	QC3 100%	**QC3 Ext**	**Recovery**
N =	4 of 4	4 of 4	
Data Point #1	14466815	11297546	78.1%
Data Point #2	14439308	11113930	77.0%
Data Point #3	14583767	11712547	80.3%
Data Point #4	14740886	12006762	81. 5%
Mean	14557694	11532696	79.2%

Prednisone

	QC1 100%	**QC1 Ext**	**Recovery**
N =	4 of 4	4 of 4	
Data Point #1	230571	167942	72.8%
Data Point #2	222306	164727	74.1%
Data Point #3	228459	166724	73.0%
Data Point #4	223172	165728	74.3%
Mean	226127	166280	73.5%
	QC2 100%	**QC2 Ext**	**Recovery**
N =	4 of 4	4 of 4	
Data Point #1	1055920	782741	74.1%
Data Point #2	1040078	801541	77.1%
Data Point #3	1054412	753180	71.4%
Data Point #4	1060692	760615	71.7%
Mean	1052776	774519	73.6%
	QC3 100%	**QC3 Ext**	**Recovery**
N =	4 of 4	4 of 4	
Data Point #1	1988749	1424481	71.6%
Data Point #2	2005401	1408663	70.2%
Data Point #3	2014388	1487534	73.9%
Data Point #4	1989498	1512129	76.0%
Mean	1999509	1458202	72.9%

Dexamethasone

	IS 100%	**IS Ext**	**Recovery**
N=	12 of 12	12 of 12	
Data Point #1	37532289	30566385	81.4%
Data Point #2	36384499	30401293	83.6%
Data Point #3	36717119	30194001	82.2%
Data Point #4	36230810	30282588	83.6%
Data Point #5	37200017	29458118	79.2%
Data Point #6	37112266	30783098	83.0%
Data Point #7	36588946	29154129	79.7%
Data Point #8	36481255	29946647	82.1%
Data Point #9	36467918	29159559	80.0%
Data Point #10	36049318	29567495	82.0%
Data Point #11	36078791	30029494	83.2%
Data Point #12	35970806	30701427	85.4%
Mean	36567836	30020353	82.1%

All values show the peak area for each analyte

8.1.3.6 Short-Term Stability in Matrix

The stability of prednisolone and prednisone in the three control samples was evaluated by the analysis of 6 replicates at each level. A set at each level was analysed as follows:

- On the day of preparation, t0
- After a minimum of 24 hours at ambient temperature.
- After a minimum of 24 hours at approximately 4°C.
- After three freeze-thaw cycles at nominal –20°C.

Prednisolone and prednisone stability data show that they are stable in these circumstances (**Table 61 & Table 62**). All the results were in the acceptable range (within ±15%).

Table 61: Prednisolone Short-Term Stability Data.

		QC1 t0	QC2 t0	QC3 t0
Batch1	**Expected Conc.(µg/L)**	**25.0**	**125.0**	**250.0**
	N =	6 of 6	6 of 6	6 of 6
	Data Point #1	24.8	110.1	260.1
	Data Point #2	26.3	135.2	252.5
	Data Point #3	25.1	140.8	232.5
	Data Point #4	23.6	138.1	255
	Data Point #5	26.0	132.1	258.3
	Data Point #6	25.8	135.4	250
	Mean	25.3	132	251.4
	Standard Dev.	1.0	11.1	9.9
	%CV	3.9	8.4	4.0
	Accuracy	101.0	105.6	100.6
		QC1 Rt	**QC2 Rt**	**QC3 Rt**
Batch2	**Expected Conc.(µg/L)**	**25.0**	**125.0**	**250.0**
	N =	4 of 4	4 of 4	4 of 4
	Data Point #1	26.6	127.8	262.9
	Data Point #2	26.2	124.9	263.1
	Data Point #3	25.9	131.2	264.2
	Data Point #4	25.5	131	267
	Mean	26.1	128.8	264.3
	Standard Dev.	0.5	3.0	1.9
	%CV	2.0	2.3	0.7
	Accuracy	104.2	103.0	105.7
		QC1 4°C	**QC2 4°C**	**QC3 4°C**
Batch2	**Expected Conc.(µg/L)**	**25.0**	**125.0**	**250.0**
	N =	4 of 4	4 of 4	4 of 4
	Data Point #1	25	126	263.6
	Data Point #2	23.8	128.9	268
	Data Point #3	24.8	129.9	271.3
	Data Point #4	25.3	127.5	274.5
	Mean	24.7	128.1	269.4
	Standard Dev.	0.6	1.7	4.7
	%CV	2.6	1.3	1.7
	Accuracy	99.0	102.5	107.7

		QC1 F/T 1	QC2 F/T 1	QC3 F/T 1
Batch2	**Expected Conc. (µg/L)**	**25.0**	**125.0**	**250.0**
	N =	4 of 4	4 of 4	4 of 4
	Data Point #1	26.9	129.6	259
	Data Point #2	26.2	130	240.2
	Data Point #3	24.9	123.1	256.9
	Data Point #4	24.5	129.6	251.7
	Mean	25.6	128.1	252
	Standard Dev.	1.1	3.3	8.4
	%CV	4.4	2.6	3.3
	Accuracy	102.5	102.5	100.8

		QC1 F/T 2	QC2 F/T 2	QC3 F/T 2
Batch3	**Expected Conc. (µg/L)**	**25.0**	**125.0**	**250.0**
	N =	4 of 4	4 of 4	4 of 4
	Data Point #1	24.6	125.6	252
	Data Point #2	24.8	124	243.4
	Data Point #3	24.7	125.6	249.3
	Data Point #4	25.7	126.2	243.8
	Mean	24.9	125.4	247.1
	Standard Dev.	0.5	0.9	4.2
	%CV	2.1	0.7	1.7
	Accuracy	99.8	100.3	98.9

		QC1 F/T 3	QC2 F/T 3	QC3 F/T 3
Batch5	**Expected Conc. (µg/L)**	**25.0**	**125.0**	**250.0**
	N =	4 of 4	4 of 4	4 of 4
	Data Point #1	26.2	113.9	247.1
	Data Point #2	24.7	114.5	244.8
	Data Point #3	23.5	123.9	246.2
	Data Point #4	22	121.1	240.3
	Mean	24.1	118.3	244.6
	Standard Dev.	1.8	4.9	3.0
	%CV	7.4	4.2	1.2
	Accuracy	96.5	94.7	97.8

t0, On the day of preparation. Rt, After 24 hours at ambient temperature. 4°C, After 24 hours at approximately 4°C. F/T1, Freeze-thaw cycle 1. F/T2, Freeze-thaw cycle 2. F/T3, Freeze-thaw cycle 3.

Table 62: Prednisone Short-Term Stability Data.

		QC1 t0	QC2 t0	QC3 t0
Batch1	**Expected Conc. (µg/L)**	**5.0**	**25.0**	**50.0**
	N =	6 of 6	6 of 6	6 of 6
	Data Point #1	5.1	21.3	50.3
	Data Point #2	5.3	26.1	48.1
	Data Point #3	5.2	27.8	45.2
	Data Point #4	4.9	27.2	47.7
	Data Point #5	5.2	26.5	49.2
	Data Point #6	5.1	27.3	47.8
	Mean	5.1	26.0	48.0
	Standard Dev.	0.1	2.4	1.7
	%CV	2.7	9.3	3.5
	Accuracy	102.5	104.1	96.1
		QC1 Rt	**QC2 Rt**	**QC3 Rt**
Batch2	**Expected Conc. (µg/L)**	**5.0**	**25.0**	**50.0**
	N =	4 of 4	4 of 4	4 of 4
	Data Point #1	5.2	23.9	49.1
	Data Point #2	5.0	23.3	49.3
	Data Point #3	5.0	24.7	49.4
	Data Point #4	5.1	24.7	49.1
	Mean	5.1	24.1	49.2
	Standard Dev.	0.1	0.7	0.1
	%CV	1.9	2.9	0.2
	Accuracy	101.9	96.6	98.4
		QC1 4°C	**QC2 4°C**	**QC3 4°C**
Batch2	**Expected Conc. (µg/L)**	**5.0**	**25.0**	**50.0**
	N =	4 of 4	4 of 4	4 of 4
	Data Point #1	5.1	24.7	51.7
	Data Point #2	4.7	25.6	51.9
	Data Point #3	4.9	25.5	52.7
	Data Point #4	5.1	25.1	53.5
	Mean	5.0	25.2	52.4
	Standard Dev.	0.2	0.4	0.8
	%CV	4.3	1.7	1.5
	Accuracy	99.2	100.9	104.9
		QC1 F/T 1	**QC2 F/T 1**	**QC3 F/T 1**
Batch2	**Expected Conc. (µg/L)**	**5.0**	**25.0**	**50.0**
	N =	4 of 4	4 of 4	4 of 4
	Data Point #1	5.6	26.3	50.9
	Data Point #2	5.3	26.3	45.6
	Data Point #3	5.0	24.8	51.8
	Data Point #4	4.9	26.3	49.0
	Mean	5.2	25.9	49.3
	Standard Dev.	0.3	0.7	2.7
	%CV	5.8	2.9	5.5
	Accuracy	103.7	103.7	98.6

		QC1 F/T 2	QC2 F/T 2	QC3 F/T 2
Batch3	**Expected Conc. (µg/L)**	**5.0**	**25.0**	**50.0**
	N =	4 of 4	4 of 4	4 of 4
	Data Point #1	4.7	23.9	47.0
	Data Point #2	4.9	23.7	43.3
	Data Point #3	5.1	23.4	45.0
	Data Point #4	5.1	23.3	45.7
	Mean	5.0	23.6	45.2
	Standard Dev.	0.2	0.3	1.5
	%CV	4.1	1.1	3.4
	Accuracy	99.3	94.3	90.5
		QC1 F/T 3	**QC2 F/T 3**	**QC3 F/T 3**
Batch4	**Expected Conc. (µg/L)**	**5.0**	**25.0**	**50.0**
	N =	4 of 4	4 of 4	4 of 4
	Data Point #1	5.1	27.8	47.9
	Data Point #2	4.8	26.7	47.4
	Data Point #3	5.7	26.2	47.9
	Data Point #4	5.2	24.1	49.5
	Mean	5.2	26.2	48.2
	Standard Dev.	0.4	1.5	0.9
	%CV	7.0	5.9	1.8
	Accuracy	103.7	104.9	96.3

t0, On the day of preparation. Rt, After 24 hours at ambient temperature. 4°C After 24 hours at approximately 4°C. F/T1, Freeze-thaw cycle 1. F/T2, Freeze-thaw cycle 2. F/T3, Freeze-thaw cycle 3.

8.1.3.7 Autosampler Stability

The stability of the extracted samples was evaluated when kept inside the autosampler for ~16 h. A full set of calibration standards assayed in duplicate, a blank sample containing internal standard, a blank sample without internal standard and the six replicates of each of the three control samples. The autosampler was operated at ambient temperature. The run was injected at time 0 and approximately 16 h later. The same calibrator set was assayed at the start and end of the stability test. Each control sample was quantified with the calibration curve injected at the same time. The mean accuracy ranged between 99.9% and 105.8% for prednisolone and between 95.5% and 105.0% for prednisone and the precision (%CV) < 9.3 for all three quality control samples. All three quality control samples were stable for at least 16 hours in the autosampler and they were in the acceptable range (**Table 63 & Table 64**).

Table 63: Prednisolone Autosampler Stability Data

Analytical Run: Batch1

Assay start date / time: 01/04/2013 18:56

	QC1	**QC2**	**QC3**
Expected Conc. (µg/L)	**25.0**	**125.0**	**250.0**
N =	6 of 6	6 of 6	6 of 6
Data Point #1	24.8	110.1	260.1
Data Point #2	26.3	135.2	252.5
Data Point #3	25.1	140.8	232.5
Data Point #4	23.6	138.1	255.0
Data Point #5	26.0	132.1	258.3
Data Point #6	25.8	135.4	250.0
Mean	25.3	132.0	251.4
Standard Dev.	1.0	11.1	9.9
%CV	3.9	8.4	4.0
Accuracy	101.0	105.6	100.6

Analytical Run: Batch1R

Assay start date / time: 02/04/2013 11:18

	QC1	**QC2**	**QC3**
Expected Conc. (µg/L)	**25.0**	**125.0**	**250.0**
N =	6 of 6	6 of 6	6 of 6
Data Point #1	24.7	111.7	256.7
Data Point #2	26.5	136.6	254.9
Data Point #3	24.6	141.4	231.9
Data Point #4	23.8	138.6	252.4
Data Point #5	26.1	130.8	253.5
Data Point #6	25.2	134.7	248.6
Mean	25.2	132.3	249.7
Standard Dev.	1.0	10.7	9.1
%CV	4.0	8.1	3.7
Accuracy	100.7	105.8	99.9

Table 64: Prednisone Autosampler Stability Data

Analytical Run: Batch1

Assay start date / time: 01/04/2013 18:56

	QC1	**QC2**	**QC3**
Expected Conc. (µg/L)	**5.0**	**25.0**	**50.0**
N =	6 of 6	6 of 6	6 of 6
Data Point #1	5.1	21.3	50.3
Data Point #2	5.3	26.1	48.1
Data Point #3	5.2	27.8	45.2
Data Point #4	4.9	27.2	47.7
Data Point #5	5.2	26.5	49.2
Data Point #6	5.1	27.3	47.8
Mean	5.1	26.0	48.0
Standard Dev.	0.1	2.4	1.7
%CV	2.7	9.3	3.5
Accuracy	102.5	104.1	96.1

Analytical Run: Batch1R

Assay start date / time: 02/04/2013 11:18

	QC1	**QC2**	**QC3**
Expected Conc. (µg/L)	**5.0**	**25.0**	**50.0**
N =	6 of 6	6 of 6	6 of 6
Data Point #1	4.9	21.5	48.9
Data Point #2	5.3	26.7	49.0
Data Point #3	5.2	28.0	45.0
Data Point #4	4.9	27.5	47.4
Data Point #5	5.3	26.5	49.0
Data Point #6	5.3	27.2	47.2
Mean	5.2	26.2	47.7
Standard Dev.	0.2	2.4	1.6
%CV	3.5	9.1	3.3
Accuracy	103.1	105.0	95.5

8.1.4 Discussion and Conclusion

All validation results obtained met the FDA guideline requirements. In this study, a very simple liquid –liquid extraction method has been developed and validated. Cortisone and prednisolone have molecular weights less than 1 amu apart and the same product ion and this makes it difficult to separate them from each other. Hence, it was difficult to measure prednisolone concentrations in renal transplant patients. This study showed that there is no interference between cortisone with prednisolone and between prednisone with hydrocortisone. However, we cannot separate cortisone from hydrocortisone. In this method, all the analytes eluted at a very short retention time, run time 3 min, which make it easy to run a large number of samples in a short period of time. This method is a sensitive method and a very small amount of plasma (100 µL) was used. HPLC‑UV methods are not an adequate for analysing low concentrations of simultaneous corticosteroids in biological fluids and require long retention time. The sensitivity, accuracy and precision achieved by this method enable us to use it for assessment of prednisolone and prednisone pharmacokinetics in renal transplant patients' samples.

In this study, we present a sensitive, simple and rapid method to be used to selectively quantitate prednisolone and prednisone in plasma from stable post- transplant patients. All the validation results met the international requirements as defined in the guideline for analytical method validation (FDA., 2001). Previously published methods using radioimmunoassay (Adachi et al., 1991) and detection by high-performance liquid chromatography (HPLC)-ultraviolet (UV) (Majid et al., 2001) or gas chromatography–mass spectrometry (GC–MS) (Shibasaki et al., 2008) have higher limits of quantification and involve difficult extraction and derivatization steps. Several of the previously reported LC–MS/MS methods do not or hardly achieve baseline separation of prednisolone and cortisol (Frerichs and Tornatore, 2004, Ionita and Akhlaghi, 2010). In this study, a simple liquid-liquid extraction which only needs 100µL of plasma has been developed and validated. Prednisolone, its metabolite prednisone and the endogenous cortisol and cortisone are very similar in structure (**Figure 59**). Most reverse phase columns (C8, C18, etc.) would result in very long run times (Majid et al.,

2001). The Hypercarb column used in this method offers the separation of highly polar compounds with closely related structures. Using Hypercarb column, prednisolone and prednisone were baseline separated from each other and from cortisol and cortisone; however the latter two were only partially separated. Compared to previously published methods, this method requires only 100µL volume of plasma, has a simple and short extraction procedure and achieves suitable chromatographic separation of the compounds of interest from each other and from endogenous corticosteroids in 3 minutes. The method developed is selective enough to distinguish between these synthetic and endogenous corticosteroids.

In addition, this study demonstrated the validity of the analytical method developed for the quantitative determination of prednisolone and prednisone in human plasma samples. The developed method is a simple and rapid analytical method with chromatographic separation in 3 minutes. Therefore, it enables us to assess prednisolone and prednisone pharmacokinetics in renal transplant patients' samples.

8.2 The Genetic Association between CYP3A5*3, CYP3A4*22, POR*28 and ABCB1 3435 and the Pharmacokinetics of Prednisolone and Prednisone.

8.2.1 Introduction

Prednisolone is a known inducer of both CYP3A and P-glycoprotein (Anglicheau et al., 2003a). It is also a substrate for both CYP3A4/3A5 and P-glycoprotein. Data obtained from a study carried out on Japanese population, suggests that individuals with the combined CYP3A5 expresser and wild-type (CC) *ABCB1 3435* genotype have lower maximum concentrations of prednisolone in the plasma (NTV, 2011).

8.2.2 Objective of the Study:

Measurement of prednisolone and prednisone concentration in samples collected for tacrolimus measurement to explore the relationship between the *CYP3A5*, *ABCB1 3435*, *CYP3A4*22* and *POR*28* genotypes and prednisolone- prednisone exposure.

8.2.3 Materials and Methods

8.2.3.1 Patients and Study Design

This study is part of a larger pharmacokinetic study; detailed information on the study participants and procedure can be found in **Chapter 4.** The daily tacrolimus dose was adjusted according to achieve whole trough blood concentrations of 8-12 µg/L during the first three months after transplantation and 5-8 µg/L thereafter.

During once and twice daily tacrolimus sample collection, five mL of blood were collected for prednisolone measurement at each time point into EDTA tubes. A series of blood samples were collected at 0, 0.5, 1, 2, 4, 6, 8, 12, 16 and 24 hours post-dose with twice daily tacrolimus samples and at 0, 1, 2, 3, 4, 6, 8, 10, 12 and 24 hours post-dose with Advagraf® samples. Plasma was prepared by centrifugation for 10 minutes at 2500 g at room temperature. Plasma samples were stored frozen at approximately -20ºC until the drug bioanalysis.

8.2.3.2 Genotyping

DNA was extracted from a peripheral blood sample using a QIAamp DNA Blood Mini Kit (Qiagen®, West Sussex, UK) and then was stored at −20◦C until analysis. *CYP3A5*3* allele was identified by performing Real-time polymerase chain reaction (RT-PCR) method (Fredericks et al., 2005). The *ABCB1* gene C and T alleles in exon 26 (C3435T) were identified using the RT-PCR method described by (Nauck et al., 2000). The C and T alleles of *POR*28* gene and in intron 6 of *CYP3A4*22* gene were identified by the new developed RT-PCR using LightCycler as described previously. Full methodology is provided in **Chapter 4** and **Chapter 5**.

8.2.3.3 Prednisolone and Prednisone Analysis in Plasma

Plasma concentration of prednisolone and its metabolite prednisone in the collected samples was analysed using the liquid chromatography-mass spectrometry (LC-MS/MS) method as described above.

8.2.3.4 Pharmacokinetic and Statistical Analysis

Prednisolone and prednisone individual pharmacokinetic parameters were determined for each genotype group. The area under the plasma concentration-time curve (AUC) was calculated using the linear trapezoidal method. The maximal plasma concentration (C_{max}) and time required to reach the peak (t_{max}) were directly obtained from the profile.

Statistical analysis was performed using Minitab statistical software (Minitab 17) to assess the statistical significance of differences in prednisolone and prednisone kinetics between different genotype groups. The log-transformed data was analysed using analysis of variance (ANOVA). Data are expressed as mean ± standard deviation (SD).

8.2.4 Results

8.2.4.1 Patient Population

Out of the sixty-four patients participating in tacrolimus study, thirty-eight renal transplant recipients on treatment with 5mg prednisolone were included in this study. However, the whole area under time concentration curve (AUC_{0-24}) was available for 27 patients because of sampling problems; some patients took their 5 mg prednisolone dose before collection of the pre-dose sample. The demographic characteristics and immunosuppression therapy are summarized in **Table 65**.

Table 65: Population Characteristics and Immunosuppression Therapy

Characteristics	**Results**
Age (yr), mean (SD)	55.4 (13.8)
Male gender, n (%)	20 (74%)
Ethnic group, n (%)	
White	17 (63%)
Black	5 (19%)
Asian	5 (19%)
Body weight (kg), mean (SD)	76.7 (14.3)
Height (cm), mean (SD)	171 (8.7)
Diabetes, n (%)	6 (22.2%)
Time since transplantation (years)	
Mean (SD)	5.5 (4.3)
Median (range)	5.2 (0.3-15.6)
Donor type, n (%)	
Living / Deceased	9 (33%) / 18 (67%)
Immunosuppression at baseline:	
Tacrolimus, n (%)	
Prograf®/ Adoport®	25 (92.4%) / 2 (7.4%)
Mycophenolate, n (%)	6 (22.2%)

8.2.4.2 Sample Analysis

During analysis of the study samples, all the batches met the acceptance criteria for this study. Samples from these batches were successfully analysed. The quality control sample data for prednisolone, prednisone obtained during the analysis are shown in **Table 66** & **Table** 67, respectively. The calibration curve data for prednisone achieved during the analysis are shown in **Table 68** & **Table 69**.

Table 66: Quality Control (QC) Samples Achieved During Prednisolone Analysis.

Quality Control Levels	**QC1**	**QC2**	**QC3**
Prepared Concentrations (µg/L)	**25.0**	**125.0**	**250.0**
Batch ID	**Measured Concentration (µg/L)**		
Batch 1	23.6	119.9	239.3
	24.3	122.7	242.1
	24.9	116.5	224.3
	24.9	116.2	228.4
Batch 2	25.1	126.7	247.8
	26.9	124.3	244.1
	25.6		
	25.9		
Batch 3	24.3	121.0	241.9
	24.8	119.3	249.7
	23.3		
	23.3		
Batch 3R	23.9	137.3	244.3
	24.4	137.8	247.4
	25.8		
	25.3		
Batch 4	24.7	125.2	239.2
	24.4	126.6	249.5
	24.8	120.1	232.5
	24.7	116.7	257.3
	24.4		237.3
	24.3		253.5
Batch 5	26.1	120.3	234.6
	25.5	119.7	235.6
	24.6	117.6	229.9
	25.7	116.6	222.0
	23.4		222.4
	22.9		222.3
Batch 6	24.8	123.9	245.3
	24.3	127.9	245.9
	24.2	129.0	242.6
	24.2	136.6	240.9
	25.6		
	25.2		

Batch 7	23.2	134.7	255.6
	24.0	138.4	250.8
	26.7	114.5	245.0
	25.4	119.0	254.2
Batch 8	25.0	133.7	265.1
	25.0	141.5	280.6
	28.7	118.7	
	28.7	113.8	
Mean (µg/L)	24.9	124.5	242.9
SD	1.2	8.1	12.8
Precision (%CV)	5.0	6.5	5.3
Accuracy (%)	99.7	99.6	97.1
N	42 of 42	30 of 30	32 of 32

Table 67: Quality Control (QC) Samples Achieved During Prednisone Analysis.

Quality Control Levels	**QC1**	**QC2**	**QC3**
Prepared Concentrations (µg/L)	**5.0**	**25.0**	**50.0**
Batch ID	**Measured Concentration (µg/L)**		
Batch 1	5.0	22.8	44.6
	4.9	23.5	43.3
	4.9	22.4	43.7
	5.1	23.6	44.6
Batch 2	4.8	22.5	43.5
	5.0	22.4	44.5
	5.0		
	5.0		
Batch 3	5.0	24.3	48.4
	4.9	24.1	49.7
	4.3		
	4.3		
Batch 3R	4.8	24.7	44.1
	5.0	25.7	44.7
	4.8		
	4.6		
Batch 4	5.2	25.5	48.1
	5.1	26.5	49.6
	5.2	24.4	45.7
	5.2	23.3	50.9
	5.0		46.6
	4.8		49.5
Batch 5	5.3	23.9	46.1
	5.2	22.9	46.7
	5.1	24.1	47.2
	5.3	23.6	46.2
	4.8		44.6
	4.7		44.6
Batch 6	5.1	24.9	47.8
	5.0	26.5	47.9
	4.5	23.0	43.2
	4.7	25.1	42.8
	4.7		
	4.6		

Batch 7	4.9	27.9	52.5
	4.9	28.7	51.8
	#5.9	26.2	51.5
	5.7	26.2	53.8
Batch 8	5.0	25.3	51.1
	4.9	25.7	#60.5
	#6.2	22.8	
	5.7	21.7	
Mean (µg/L)	5.0	24.5	47.1
SD	0.3	1.7	3.1
Precision (%CV)	5.8	6.9	6.6
Accuracy (%)	99.1	97.9	94.1
N	40 of 42	30 of 30	31 of 32

#excluded from the quality controls used (>15% deviation from its actual concentration)

Table 68: Calibration Curve Parameters Data Achieved During Prednisolone Analysis.

Batch ID	Curve parameters ($y=a2x^2+a1x+a0$)			
	a0	a1	a2	Correlation Coefficient (r)
Batch1	0.00018	0.00197	-7.84E-07	0.9996
Batch2	0.00249	0.00177	-5.25E-07	0.9954
Batch3	0.00079	0.00189	-8.44E-07	0.9989
Batch3R	0.00040	0.00176	-7.33E-07	0.9987
Batch4	0.00077	0.00188	-9.34E-07	0.9992
Batch5	0.00068	0.00184	-6.42E-07	0.9997
Batch6	0.00157	0.00180	-8.39E-07	0.9994
Batch7	0.00074	0.00185	-1.19E-06	0.9993
Batch8	0.00088	0.00116	-1.45E-07	0.9998

Table 69: Calibration Curve Parameters Data Achieved During Prednisone Analysis.

Batch ID	Curve parameters (y = A*x + B)		
	Slope (A)	Intercept (B)	Correlation Coefficient (r)
Batch1	0.00102	3.84E-05	0.9989
Batch2	0.00096	0.000188	0.9966
Batch3	0.00112	0.000113	0.9989
Batch3R	0.00109	0.000111	0.9989
Batch4	0.00101	0.000117	0.9993
Batch5	0.000999	5.9E-05	0.9995
Batch6	0.00101	0.000176	0.9982
Batch7	0.00092	0.000121	0.9964
Batch8	0.00069	0.000104	0.9989

The time–concentration profiles for prednisolone and prednisone mean blood concentrations are shown in **Figure 63**. The individual time–concentration profiles of the 27 patients are presented in **Figure 64.** The prednisolone and prednisone pharmacokinetic parameters, C_{max}, AUC_{0-24} and C_0 are summarised in **Table 70**. The between-patient coefficients of variation (CV%) of C_{max}, AUC_{0-24} and C_0 for prednisolone were 24.4%, 23.2% and 55.6%, respectively. The between-patient coefficients of variation (CV%) of C_{max}, AUC_{0-24} and C_0 for prednisone were 24.2%, 24.9% and 73.0%, respectively. Prednisolone and prednisone exposure $AUC_{0–24}$, C_{max} and C_0 of the individual patients are presented in **Figure 65**.

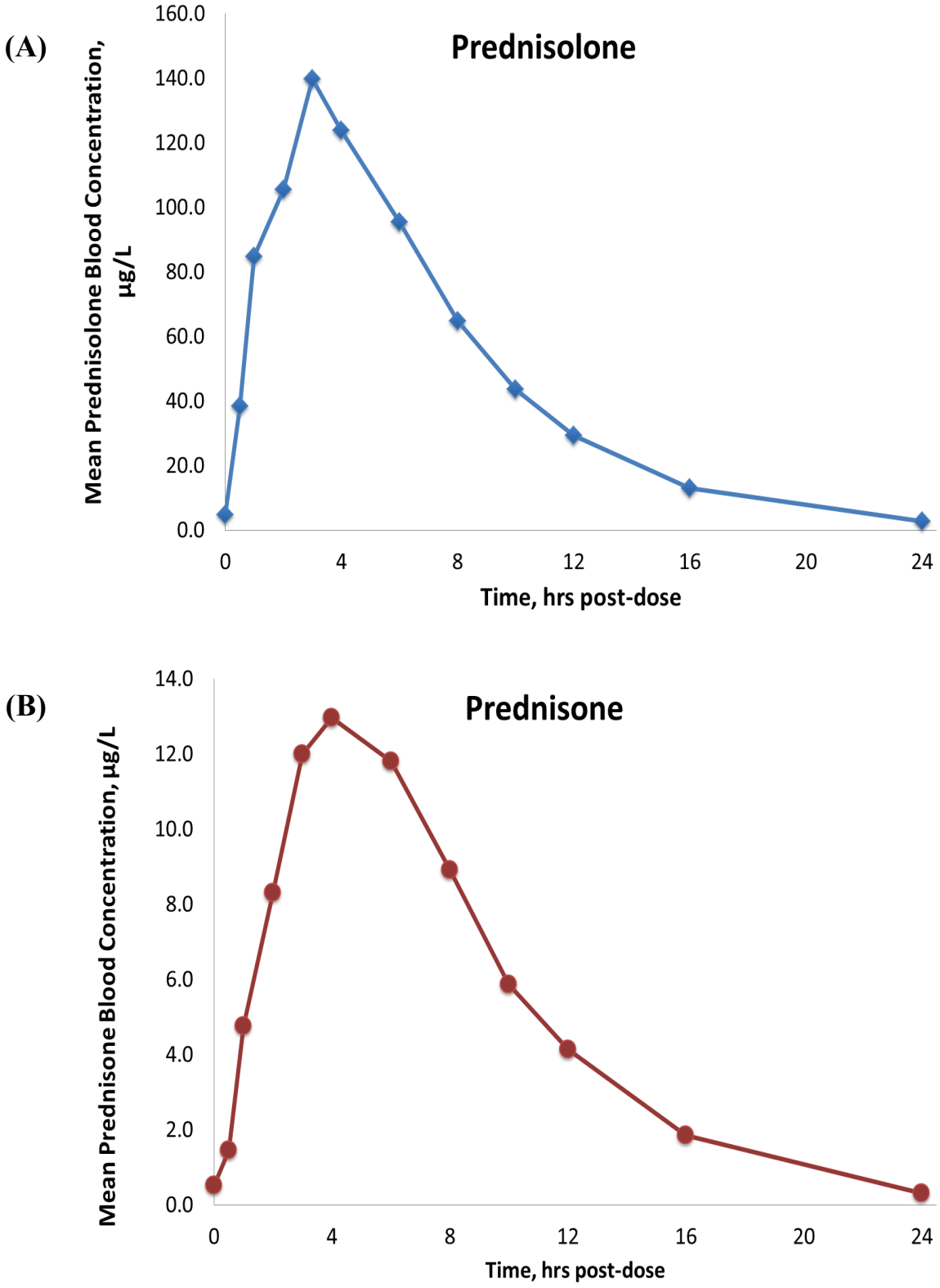


Figure 63: Mean Blood Concentration-Time Profiles for Prednisolone (A) and Prednisone (B) in 27 Stable Kidney Transplant Recipients.

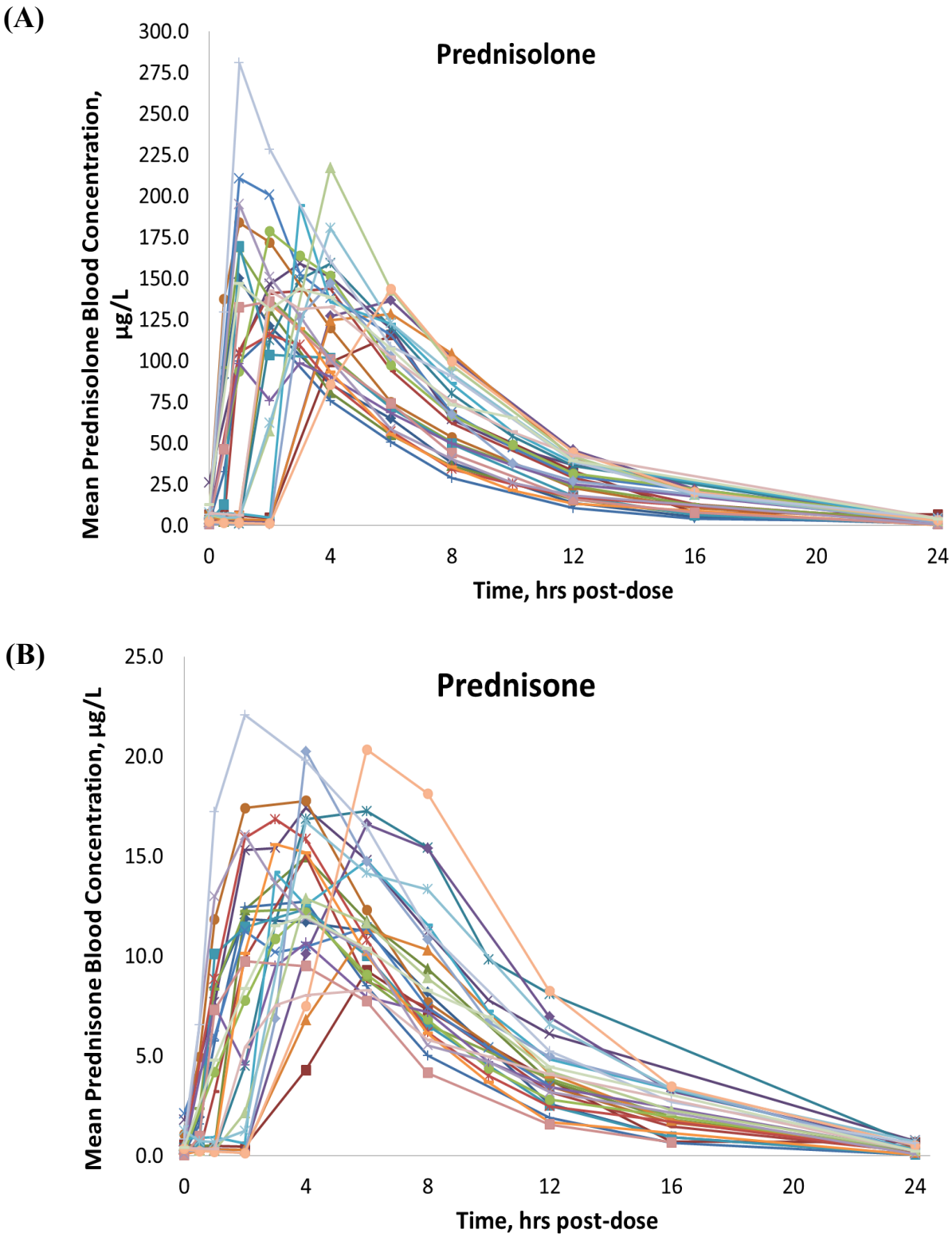


Figure 64: Whole-Blood Concentration-Time Profiles for Prednisolone (A) and Prednisone (B) in 27 Stable Kidney Transplant Recipients.

Table 70: Prednisolone-Prednisone Pharmacokinetic Parameters for Individual Patients Treated with 5mg Prednisolone.

Patient	C_{max} (µg/L)		AUC_{0-24} (µg∗h/L)		C_0 (µg/L)	
	Prednisolone	**Prednisone**	**Prednisolone**	**Prednisone**	**Prednisolone**	**Prednisone**
1	150	11.9	844.5	112.2	1.0	0.1
2	115.6	9.3	1066.9	93.4	4.6	0.5
3	130.6	15.0	690.9	127.8	1.3	0.3
4	159.3	17.4	1396.6	180.4	4.5	0.8
5	159.1	17.3	1281.7	187.2	1.5	0.3
6	184	17.8	1183.9	146.6	2.7	0.4
7	116.8	12.7	683.5	93.2	1.2	0.1
8	143.9	15.0	1159.1	109.7	2.0	0.2
9	166.7	12.4	1068.5	116.3	3.2	0.3
10	136.7	16.6	1177.8	149.9	2.8	0.3
11	169.6	12.4	898.5	105.4	1.0	0.1
12	128.5	11.4	1130.0	96.0	2.7	0.2
13	210.8	11.4	1467.6	100.3	1.8	0.1
14	116.0	16.9	823.6	126.1	1.4	0.2
15	178.7	12.2	1332.1	102.9	2.7	0.1
16	98.7	10.7	909.1	102.9	1.8	0.2
17	194.5	14.8	1227.8	137.8	2.9	0.3
18	134.8	15.6	722.9	100.9	0.4	0.1
19	147.2	20.3	965.2	138.7	1.0	0.2
20	136.1	9.7	922.2	77.9	0.8	0.04
21	217.3	12.9	1398.1	111.3	2.9	0.3
22	195.1	16.1	1008.8	124.6	0.9	0.1
23	180.7	16.7	1275.9	154.3	4.3	0.7
24	143.7	20.3	1076.7	166.6	1.3	0.1
25	280.8	22.1	1722.1	195.5	4.5	0.6
26	142.6	8.3	1277.7	93.8	4.4	0.2
27	147.1	12.0	1428.3	126.6	4.1	0.2
Mean	160.5	14.5	1116.3	125.1	2.4	0.3
SD	39.1	3.5	259.1	31.1	1.3	0.2
CV%	24.4	24.2	23.2	24.9	55.6	73.0

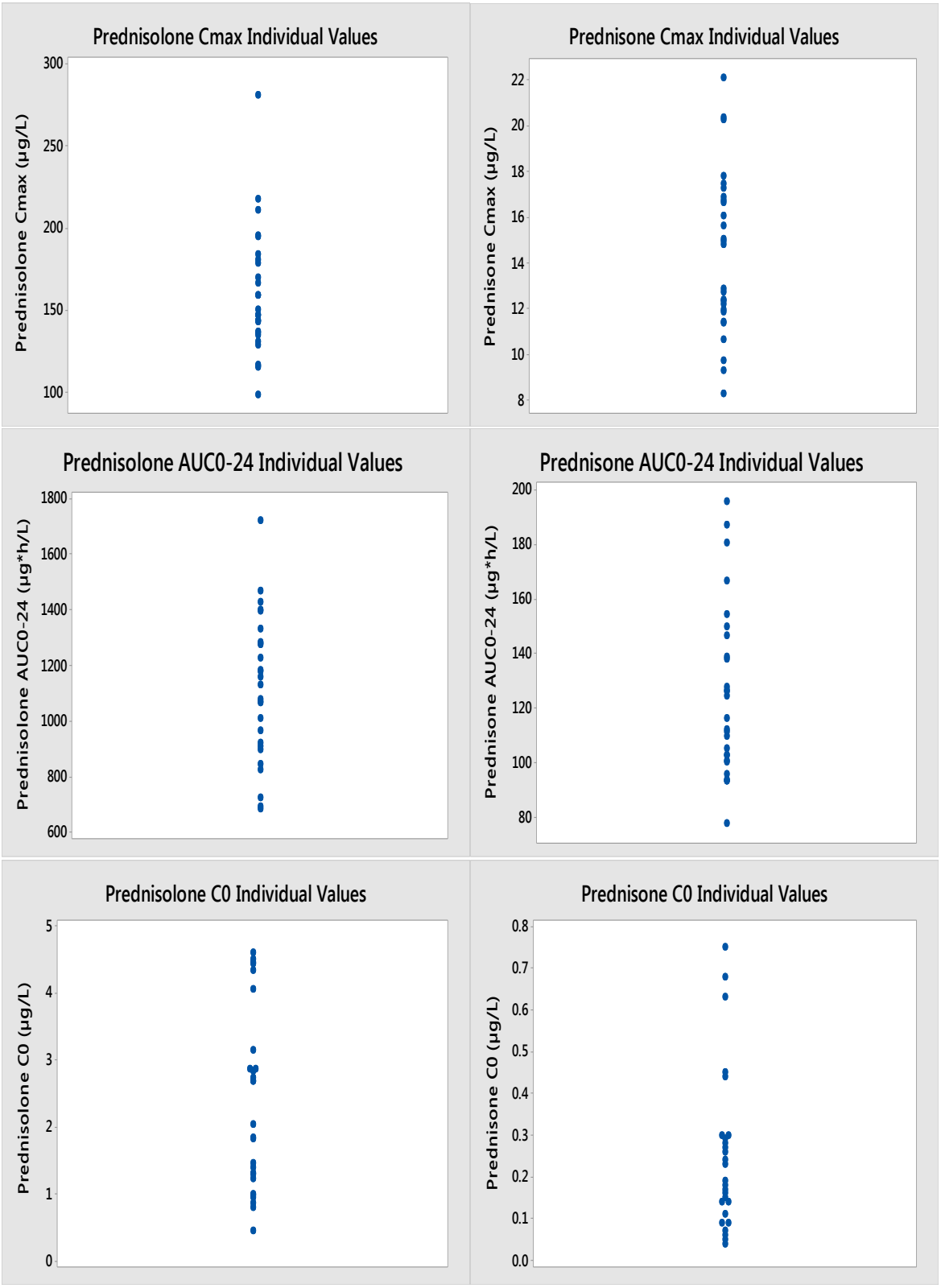


Figure 65: Individual Value Plot of Prednisolone and Prednisone AUC_{0-24}, C_{max} and C_0 for Patients Treated with 5mg Prednisolone.

There was a good correlation between $AUC_{0–24}$ and trough concentrations (C_0) for prednisolone and prednisone at steady state, with similar correlation coefficients for prednisolone and prednisone (r = 0.71 and r = 0.64, respectively); see **Figure 66.** Moreover, there was a strong correlation between $AUC_{0–24}$ and C_{max} for prednisolone and prednisone at steady state (r = 0.70 and r = 0.84, respectively); see **Figure 67**.

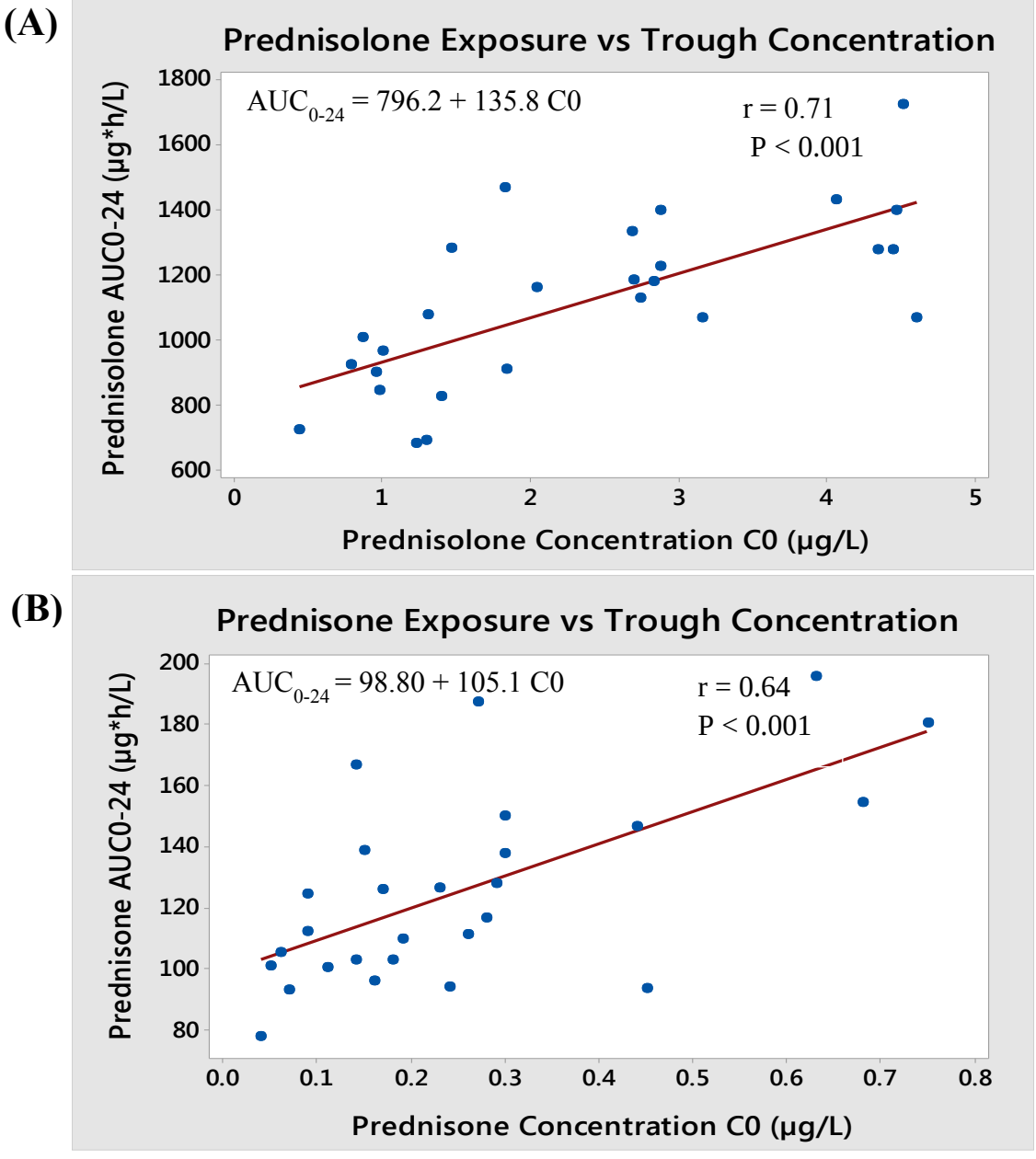
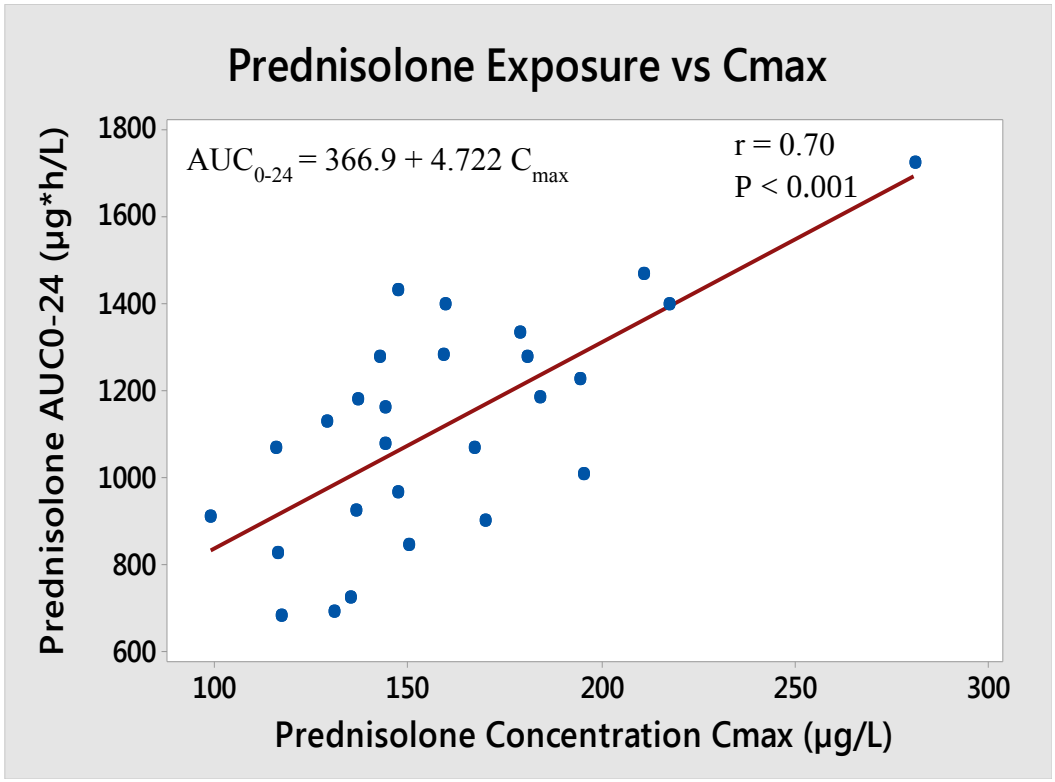


Figure 66: Correlation between Prednisolone (A) and Prednisone (B) Exposure and Trough Concentration using Regression Analysis (Fit Regression Model).



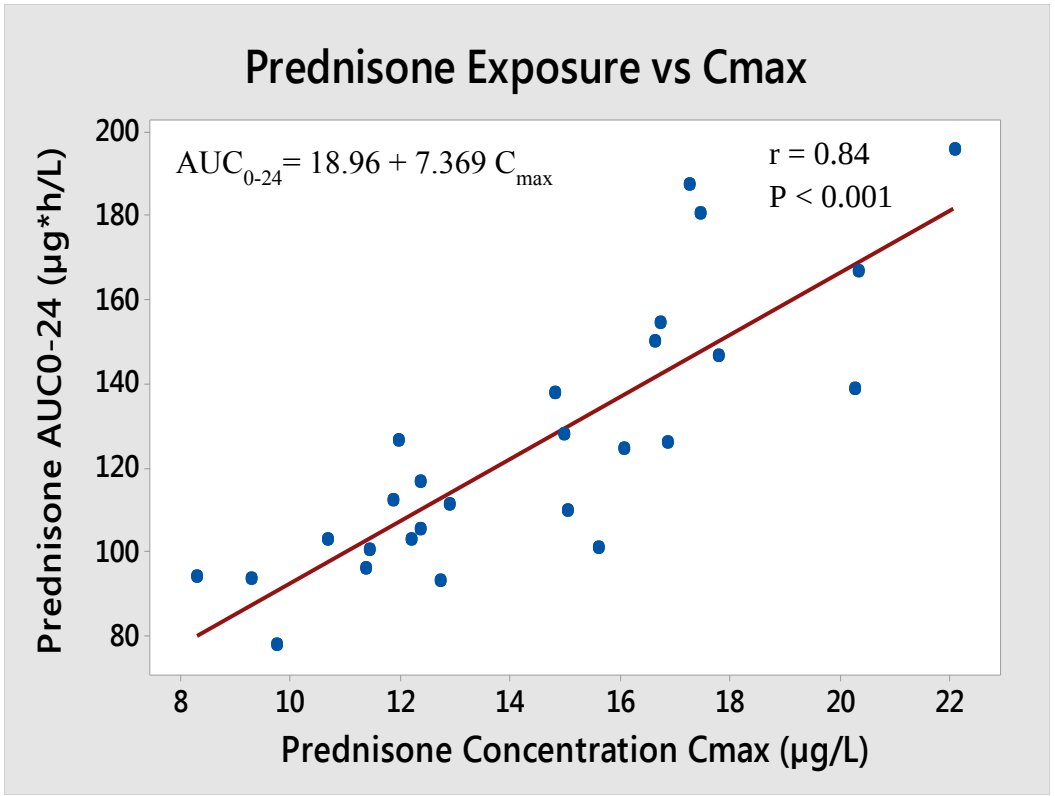


Figure 67: Correlation between Prednisolone (A) and Prednisone (B) Exposure and Maximum Concentration using Regression Analysis (Fit Regression Model).

*8.2.4.3 The Relationship between CYP3A5*3 Genotype and Prednisolone-Prednisone Pharmacokinetics.*

Prednisolone and prednisone blood concentrations C_{max}, AUC_{0-24} and C_0 showed a log-normal distribution and the data were therefore log-transformed before analysis. In the overall study population, no significant association was observed between the different CYP3A5 genotypes with prednisolone pharmacokinetic parameters; $AUC_{0–24,}$ C_{max} and trough concentration (C_0). Dissimilar to the prednisolone results, there were significant differences in the mean $AUC_{0–24}$ and C_{max} of prednisone between these genotype groups. The mean prednisone C_{max} and $AUC_{0–24}$ in recipients having the *CYP3A5*3/*3* genotype were significantly lower than in those patients having the *CYP3A5*1/*1 + *1/*3* genotype (P = 0.006 and P = 0.027, respectively). However, there was no significant change in prednisone C_0 between CYP3A5 expressers and non-expressers. After adjustment for the body weight, we continued to have the same statistically significant results. The pharmacokinetic parameters in recipients with the *CYP3A5*1/*1 + *1/*3* and *CYP3A5*3/*3* genotypes for prednisolone and prednisone are shown in **Table 71** & **Figure 68**.

Table 71: Pharmacokinetic Parameters of Prednisolone-Prednisone in *CYP3A5* Genotype Groups

Genotype group	CYP3A5 Expressers (*1/*1 & *1/*3) (n= 12)	CYP3A5 Non-expressers (*3/*3) (n= 15)	p-value
Prednisolone			
C_{max} (µg/L)	170.2 ± 43.5	149.5 ± 32.8	0.13
AUC_{0-24} (µg*h/L)	1170.6 ± 278.7	1072.8 ± 243.1	0.32
C_0 (µg/L)	2.3 ± 1.2	2.4 ± 1.5	0.75
Prednisone			
C_{max} (µg/L)	16.4 ± 3.4	12.8 ± 2.8	0.006
AUC_{0-24} (µg*h/L)	139.3 ± 31.7	113.7 ± 26.5	0.027
C_0 (µg/L)	0.29 ± 0.19	0.21 ± 0.19	0.16

The values are shown as the mean ± S.D. C_{max}, Maximum plasma concentration; $AUC_{0–24}$, Area under the plasma concentration–time curve from 0 to 24h. C_0, Pre-dose plasma concentration at 24h. Values were compared using ANOVA (General linear model).

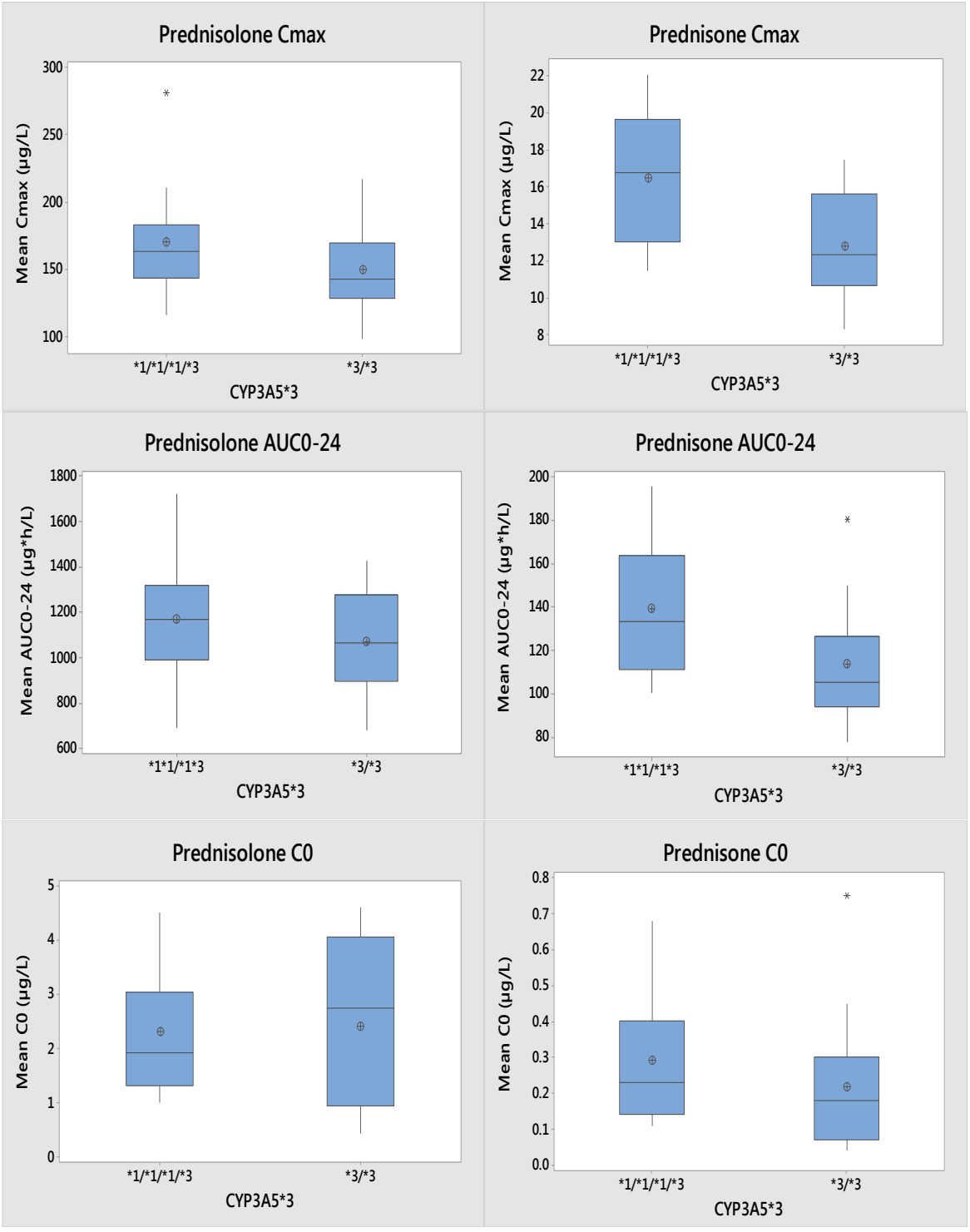


Figure 68: The Mean Prednisolone-Prednisone Pharmacokinetic Parameters in Renal Transplant Recipients with Different *CYP3A5*3* Genotypes. Boxes represent the interquartile range, line in the box represents the median value, symbol in the box represents the mean value, outer lines represent the minimal and maximal value and the values beyond the whiskers (asterisks) are outliers.

8.2.4.4 The Association between ABCB1 3435 Genotype and Prednisolone - Prednisone Pharmacokinetics.

The prednisolone-prednisone pharmacokinetic parameters stratified by *ABCB1 3435* polymorphisms are shown in **Table 72**. No significant difference was found in the $AUC_{0–24}$, C_{max} and C_0 of prednisolone between the different genotypes of *ABCB1 3435* gene. However, a significant difference was observed in prednisone pharmacokinetic parameters ($C_{max,}$ AUC_{0-24} and C_0) between the *ABCB1 CC* and the *CT/TT* genotypes. The mean prednisone C_{max} for *ABCB1* CC genotype was significantly higher than for *ABCB1 CT/TT* genotype (16.8 µg/L versus 13.7 µg/L, P = 0.04). Moreover, *ABCB1* CC carriers had a significantly higher $AUC_{0–24}$ compared to *ABCB1 CT/TT* carriers (153.9 µg*h/L versus 116.9 µg*h/L, P = 0.005). Likewise, the difference in prednisone C_0 between *ABCB1*genotype groups was statistically. The mean prednisone C_0 for *ABCB1*CC carriers and for *CT/TT* patients were 0.37 ± 0.14 and 0.22 ± 0.19 µg/L, respectively (**Figure** 69 **& Table 72**). After adjustment for the body weight, we continued to have the same statistically significant results.

Table 72: Pharmacokinetic Parameters of Prednisolone- Prednisone in *ABCB1 3435* Genotype Groups.

Study group	*ABCB1 CC* Carriers (n= 6)	*ABCB1 CT/TT* Carriers (n= 21)	p-value
Prednisolone			
C_{max} (µg/L)	176.3 ± 54.8	153.7 ± 32.6	0.08
AUC_{0-24} (µg*h/L)	1187.0 ± 333.0	1095.9 ± 239.9	0.22
C_0 (µg/L)	2.7 ± 1.2	2.3 ± 1.4	0.30
Prednisone			
C_{max} (µg/L)	16.8 ± 3.2	13.7 ± 3.4	0.04
AUC_{0-24} (µg*h/L)	153.9 ± 31.6	116.9 ± 26.3	0.005
C_0 (µg/L)	0.37 ± 0.14	0.22 ± 0.19	0.03

Values were compared using ANOVA (General linear model).

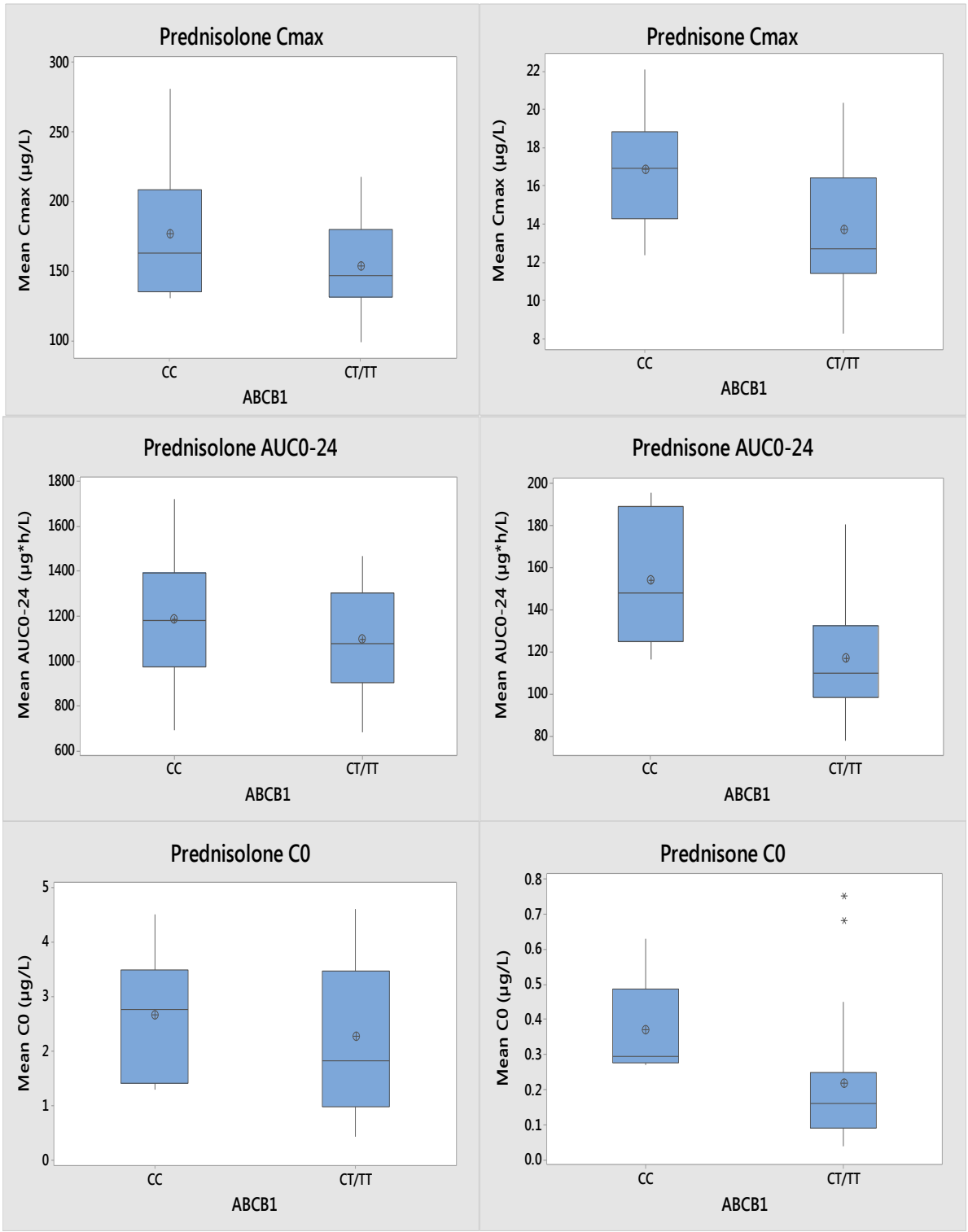


Figure 69: The Mean Prednisolone-Prednisone Pharmacokinetic Parameters in Renal Transplant Recipients with Different *ABCB1* Genotypes. Boxes represent the interquartile range, line in the box represents the median value, symbol in the box represents the mean value, outer lines represent the minimal and maximal value and values beyond the whiskers (asterisks) are outliers.

*8.2.4.5 Association of CYP3A5*3 and ABCB1 3435 Combined Genotypes with Prednisolone and Prednisone Pharmacokinetics.*

Prednisolone and prednisone pharmacokinetic parameters in the two different CYP3A5 genotype groups in relation to *ABCB1 3435* polymorphisms are shown in **Table 73**. Because there was only one patient in CYP3A5 **3/*3* / *ABCB1 CC* group, it was excluded from the analysis. Regarding prednisolone, no significant difference was found in prednisolone C_{max}, AUC_{0-24} and C_0 between the three genotype groups. However, the mean C_{max} and AUC_{0-24} of prednisone in CYP3A5 **3/*3* patients having the *ABCB1 CT/TT* genotype were lower than in CYP3A5 **1/*1&*1/*3* patients having either *ABCB1 CC* or *ABCB1 CT/TT* genotypes. No significant difference was noted in CYP3A5 expressers between *ABCB1 CT/TT* and *ABCB1CC* carriers for prednisolone and prednisone **Figure 70**. After adjustment for the body weight, we continued to have the same statistically significant results.

Table 73: Pharmacokinetic Parameters of Prednisolone- Prednisone in *CYP3A5-ABCB1 3435* Genotype Groups.

Study group	*CYP3A5 *1/*1 &*1/*3/ ABCB1 CC* Carriers (n= 5)	*CYP3A5 *1/*1 &*1/*3/ ABCB1 CT/TT* Carriers (n= 7)	*CYP3A5 *3/*3/ ABCB1 CT/TT* Carriers (n= 14)	p-value [a]	p-value [b]	p-value [c]
Prednisolone						
C_{max} (µg/L)	184.2 ± 57.3	160.1 ± 31.6	150.4 ± 33.8	0.37	0.12	0.52
AUC_{0-24} (µg*h/L)	1189 ± 373	1157 ± 222	1065 ± 250.5	0.99	0.48	0.43
C_0 (µg/L)	2.6 ± 1.3	2.1 ± 1.1	2.4 ± 1.5	0.56	0.42	0.97
Prednisone						
C_{max} (µg/L)	16.9 ± 3.6	16.1 ± 3.5	12.5 ± 2.7	0.55	0.01	0.03
AUC_{0-24} (µg*h/L)	155 ± 35	128 ± 26	111 ± 25	0.10	0.004	0.17
C_0 (µg/L)	0.4 ± 0.2	0.2 ± 0.2	0.2 ± 0.2	0.15	0.04	0.58

[a] p-values refer to comparisons between *CYP3A5 *1/*1& *1/*3* (CYP3A5 Expressers) subgroups.

[b] p-values refer to comparisons between *CYP3A5 *1/*1& *1/*3* / *ABCB1 CC* carriers and *CYP3A5 *3/*3* (CYP3A5 Non-expresser) /*ABCB1 CT/TT* group.

[c] p-values refer to comparisons between *CYP3A5 *1/*1& *1/*3* / *ABCB1 CT/TT* carriers and *CYP3A5 *3/*3* /*ABCB1 CT/TT* group.

Values were compared using ANOVA (General linear model).

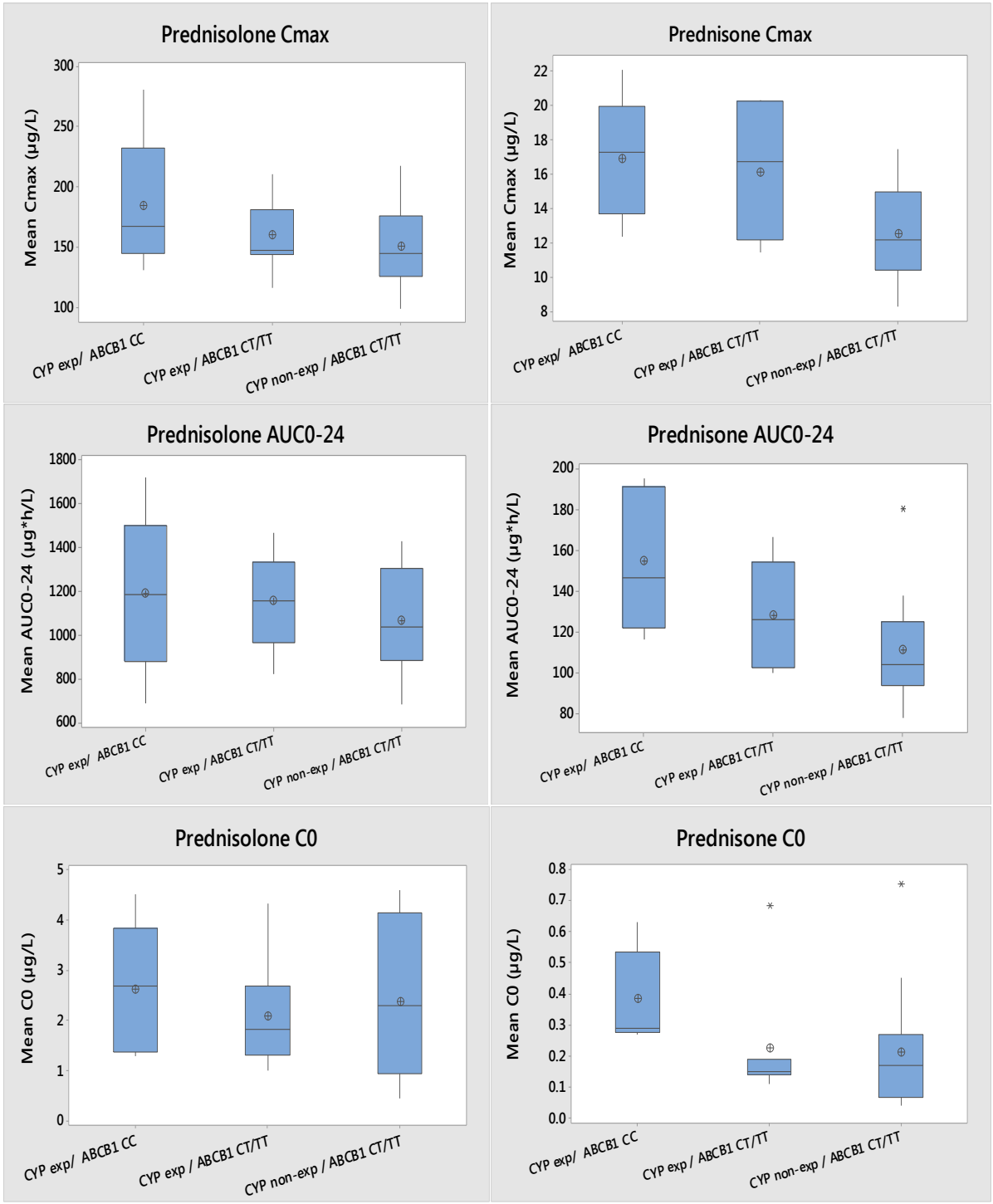


Figure 70: The Mean Prednisolone-Prednisone Pharmacokinetic Parameters in Renal Transplant Recipients with Combined *CYP3A5*3-ABCB1* Genotypes. CYP exp is CYP3A5 expressers and CYP non-exp is CYP3A5 non-expressers. Boxes represent the interquartile range, line in the box represents the median value, symbol in the box represents the mean value, outer lines represent the minimal and maximal value and values beyond the whiskers (asterisks) are outliers.

*8.2.4.6 The Relationship between POR*28 and Prednisolone and Prednisone Pharmacokinetics.*

No statistically significant differences in prednisolone pharmacokinetics were observed between patients carrying *POR*28 CC* and *CT/TT* genotypes (**Table 74**). The mean Prednisolone C_{max} for *POR*28 CC* carriers and for *CT/TT* patients were 169.0 ± 47.0 and 161.2 ± 35.0 µg/L, respectively. The mean Prednisolone $AUC_{0–24}$ for *POR*28 CC* and for *CT/TT* carriers were 1063 ± 304 µg*h/L, 1084.3 ± 279 µg*h/L and 1158 ± 219 µg*h/L, respectively. Similarly, we found no difference in prednisone pharmacokinetic profile between *POR*28* different genotypes (P > 0.05, **Figure 71**).

Table 74: Pharmacokinetic Parameters of Prednisolone- Prednisone in *POR*28* Genotype Groups.

Study group	*POR*28 CC* Carriers (n= 13)	*POR*28 CT/TT* Carriers (n= 14)	p-value
Prednisolone			
C_{max} (µg/L)	161.9 ± 46.5	155.7 ± 31.1	0.56
AUC_{0-24} (µg*h/L)	1095 ± 312	1136 ± 209	0.78
C_0 (µg/L)	2.2 ± 1.4	2.5 ± 1.3	0.61
Prednisone			
C_{max} (µg/L)	14.9 ± 3.8	14.0 ± 3.3	0.51
AUC_{0-24} (µg*h/L)	128.7 ± 36.6	121.8 ± 26.1	0.66
C_0 (µg/L)	0.28 ± 0.26	0.22 ± 0.10	0.82

Values were compared using ANOVA (General linear model).

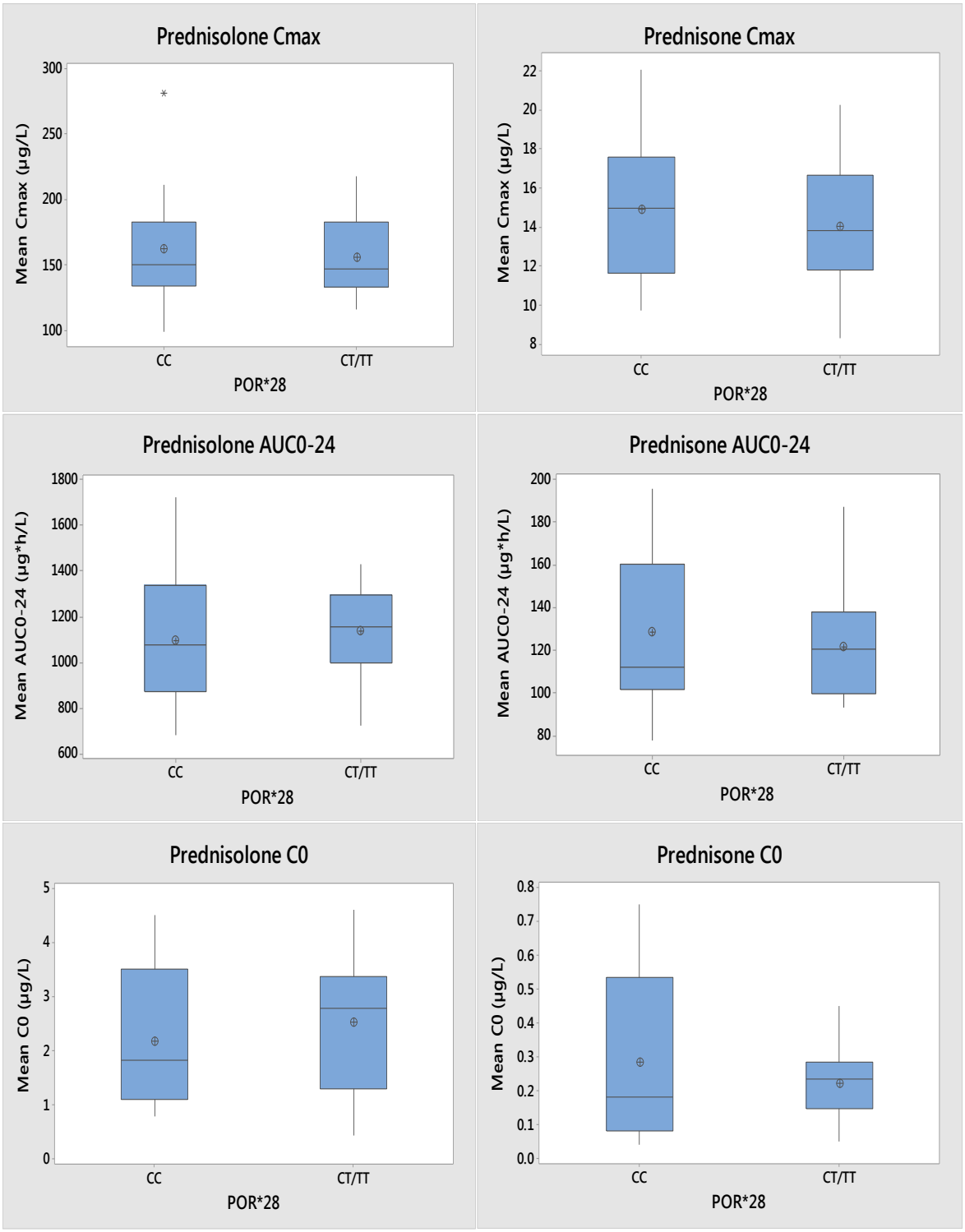


Figure 71: The Mean Prednisolone-Prednisone Pharmacokinetic Parameters in Renal Transplant Recipients with *POR*28* Different Genotypes. Boxes represent the interquartile range, line in the box represents the median value, symbol in the box represents the mean value, outer lines represent the minimal and maximal value and values beyond the whiskers (asterisks) are outliers.

8.2.4.7 Non-genetic Factors Associated with Prednisolone and Prednisone Plasma Concentrations.

No correlation was seen among different ethnic backgrounds for prednisolone or prednisone blood concentrations. Moreover, patient sex was shown to influence prednisolone $C_{max,}$ but have no effect on the AUC_{0-24} and the trough concentration (**Table 75**). In the current study, twenty-seven patients were analysed; Twenty of them were males, and they all had a significantly lower prednisolone C_{max} compared to females ($p<0.05$; **Figure 72**). After adjustment for the body weight, the statistically significant difference vanished. In addition, there was no correlation between patient age and prednisolone and prednisone blood concentrations. Multiple regression analysis by stepwise selection; alpha to enter or remove, specified value to enter or remove significance level, was 0.15; identified patient weight and tacrolimus dose, as independent variables associated with prednisolone C_{max} and identified tacrolimus dose as independent variables associated with prednisolone AUC_{0-24}. However, multiple regression analysis for prednisone identified patient weight as an independent variable associated with prednisone C_{max} and patient age and weight for prednisone AUC_{0-24}.

Table 75: Prednisolone and Prednisone Pharmacokinetic Parameters in Renal Transplant Recipients in Relation to Ethnicity and Sex.

Study group	Prednisolone		
	C_{max} (µg/L)	AUC_{0-24} (µg*h/L)	C_0 (µg/L)
Ethnicity			
Black (n=5)	170.7 ± 64.9	1117 ± 407	2.4 ± 1.4
White (n=17	157.5 ± 34.8	1125 ± 249	2.4 ± 1.3
Asian (n=5)	150.7 ± 19.0	1085 ± 148	2.0 ± 1.5
p-value	0.56	0.81	0.73
Sex			
Male (n=20)	147.6 ± 28.1	1066 ± 243	2.3 ± 1.3
Female (n=7)	190.4 ± 48.8	1260 ± 266	2.6 ± 1.5
p-value	0.04	0.28	0.78
Study group	**Prednisone**		
	C_{max} (µg/L)	AUC_{0-24} (µg*h/L)	C_0 (µg/L)
Ethnicity			
Black (n=5)	16.7 ± 3.6	151 ± 38	0.33 ± 0.18
White (n=17	13.5 ± 2.4	118 ± 25	0.24 ± 0.21
Asian (n=5)	15.3 ± 5.8	125 ± 37	0.20 ± 0.15
p-value	0.25	0.14	0.40
Sex			
Male (n=20)	14.2 ± 3.5	125.1 ± 29.2	0.24 ± 0.17
Female (n=7)	15.0 ± 4.0	125.2 ± 38.7	0.29 ± 0.25
p-value	0.74	0.83	0.80

Values were compared using ANOVA (General linear model).

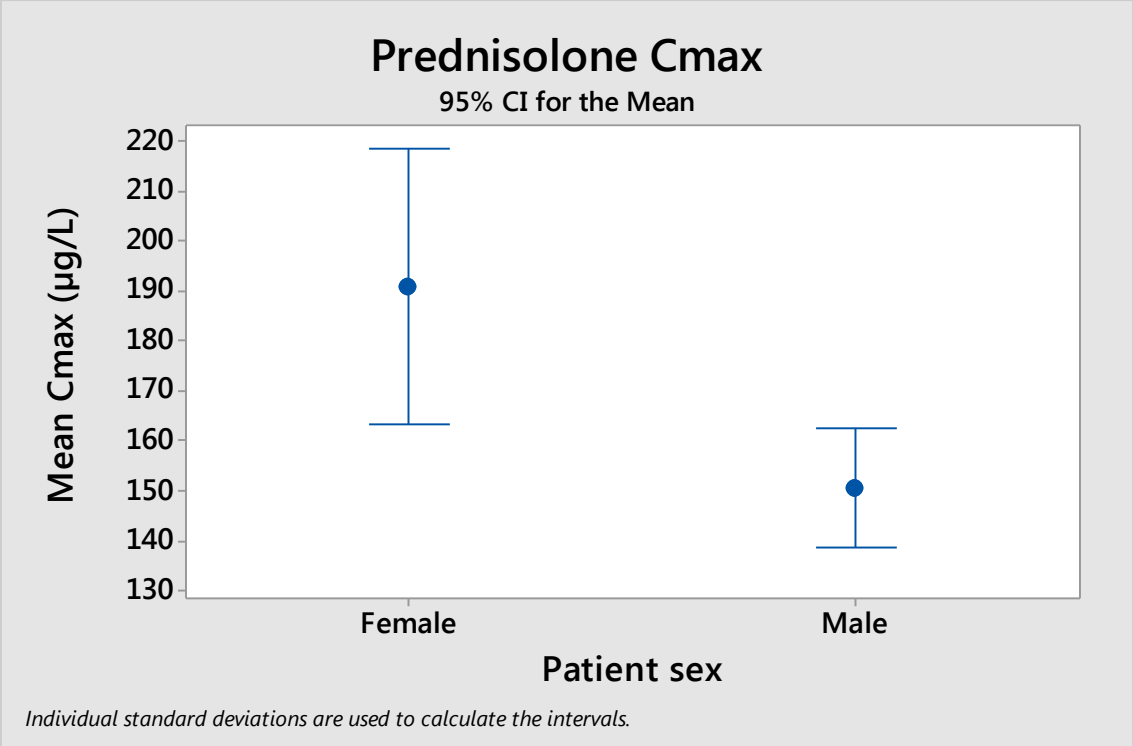


Figure 72: The Interval Plot of the Mean Prednisolone C_{max} in Renal Transplant Recipients in Accordance with Patient Sex.

8.2.5 Discussion

In this study, we determined the pharmacokinetic parameters of prednisolone and prednisone in 38 renal transplant recipients. Due to some sampling problems, some patients took their prednisolone dose before collection of the pre-dose sample; the $AUC_{0\text{-}24}$ was available for only 27 patients. Prednisolone is known as a substrate of CYP3A and P-glycoprotein (Anglicheau et al., 2003a). To improve the treatment of kidney transplant recipients, genetic markers that predict steroid response might be useful.

In this study, we found a wide variation between patients in prednisolone and prednisone blood concentration achieved by 5 mg prednisolone daily. This is in agreement with the recent study by Saeves et al. (2012) in 16 liver transplant recipients early after transplantation. Their findings demonstrated large within- and between-individual variabilities in prednisolone and prednisone pharmacokinetics, indicating the possible need for personalizing prednisolone and prednisone dosing in organ

transplantation. The same findings were obtained from pharmacokinetic study after single dose administration of prednisone by showing high between-individual variability in systemic exposure to prednisolone in systemic lupus erythematosus patients (Sagcal-Gironella et al., 2011). Similarly, Morton et al. (2006) found a wide between-individual variation in prednisolone pharmacokinetics among 52 lung transplant recipients. The majority of the patients were overdosed on the conventional protocols, suggesting the use of TDM to optimize prednisolone dosing and minimize morbidity. Moreover, Barraclough et al. (2011b) reported wide between-subject variability in prednisolone exposure in kidney transplant patients, justifying a role for TDM and suggested the use of limiting sampling strategies (LSSs) for accurate estimation of both total and free prednisolone AUC_{0-12}. Hence, to achieve a balance between toxicity and efficacy of prednisolone, therapeutic drug monitoring may be useful and would be worthy of a clinical trial.

In our data, prednisolone and prednisone behaved differently. Although the *CYP3A5* genotype plays an important role in drug metabolism, including prednisolone, no association between *CYP3A5* genetic polymorphisms and prednisolone pharmacokinetics was observed. In addition, no significant differences were found in prednisolone pharmacokinetics for *ABCB1 3435* polymorphisms. This finding is in agreement with the previous report by Miura et al. (2008), Miura et al. (2009) and showed that prednisolone is unaffected by either the *CYP3A5**3 or *ABCB1 3435* genetic polymorphisms. Moreover, *POR*28* gene was not associated with prednisolone pharmacokinetics.

In addition, the AUC_{0-24} and C_{max} of prednisolone showed no significant difference among CYP3A5 expressers having either *ABCB1 3435 CC* or *CT/TT* genotypes and CYP3A5 non-expressers having *ABCB1 3435 CT/TT* genotype. This is in agreement with Miura et al. (2008) who found no significant difference in prednisolone AUC_{0-24}, C_{max} and C_0 in *CYP3A5* expressers having either *ABCB1 3435 CC* , CT or TT alleles. They also found a significant increase in prednisolone C_{max} in CYP3A5 non-expressers having *ABCB1 3435 CC* allele compared to *ABCB1 3435 TT* carriers. They also concluded that the intestinal CYP3A5 and P-glycoprotein may play important roles in

prednisolone absorption but they have low contribution to prednisolone pharmacokinetics. However, we could not make this comparison in our study cohort because of the limited number of patients in the *CYP3A5*3/*3 / ABCB1 3435 CC* group. Moreover, we found no correlation between the different ethnic backgrounds and prednisolone blood concentrations. This indicates that neither the genetic factors nor ethnicity can predict prednisolone plasma concentration. However, patient sex had a significant effect on prednisolone C_{max}.

Similar to prednisolone, prednisone is also known as a substrate and inducer of CYP3A enzymes and P-glycoprotein (Anglicheau et al., 2003a); however, prednisone pharmacokinetics differed significantly between *CYP3A5* genotypes, suggesting preferential generation of prednisone as a metabolite in CYP3A5 expressers. Like prednisone, 20β- dihydroprednisolone was one of the main metabolites of prednisolone in a recent study by Matabosch et al. (2015) who identified 20 metabolites of prednisolone in urine samples of two healthy male Caucasian volunteers following administration of 10 mg prednisolone. Interestingly, the maximum prednisolone concentrations were similar in the excretion profiles of prednisolone metabolites in both volunteers. However, 20β- dihydroprednisolone maximum concentration was similar to prednisone maximum concentration in one volunteer and higher in the other one despite that they are both males and Caucasians. This may explain prednisone low concentration in CYP3A5 non-expressers, suggesting that 20β- dihydroprednisolone could be produced more than prednisone in CYP3A5 non-expressers. Additionally, our results showed that *ABCB1 3435* gene was strongly associated with prednisone pharmacokinetics. Conversely, *POR*28* was not associated with prednisone PK. Our results showed that patients carrying at least one *CYP3A5*1* allele had a significantly higher mean prednisone C_{max} and AUC_{0-24} compared to *CYP3A5*3* homozygotes. Similarly, we found that *ABCB1 3435 CC* carriers achieved higher mean prednisone C_{max} and AUC_{0-24} than *ABCB1 3435 CT/TT* Carriers. This finding may be due to over-representation of CYP3A5 expressers in the *ABCB1 3435 CC* group. When combining *CYP3A*5 and *ABCB1 3435* genotypes, we found a significantly lower prednisone C_0 and C_{max} for CYP3A5 non-expresser patients having the *ABCB1 3435 CT/TT* genotype compared to CYP3A5 expresser patients having *ABCB1 3435 CC* genotype. Similar

results were found in prednisone AUC_{0-24} when compared to *ABCB1 3435 CC* carriers, but not when compared to *ABCB1 3435 CT/TT* carriers. The difference in prednisone C_0, C_{max} and AUC_{0-24} between CYP3A5 expressers subgroups (*ABCB1 3435 CC* or *ABCB1 3435 CT/TT* carriers) was not statistically significant, confirming the predominant influence of CYP3A5 in prednisone exposure.

With regard to *CYP3A5* polymorphisms, we expected the prednisone C_{max} and AUC_{0-24} to be unaffected like in prednisolone or to be lower in the CYP3A5 expressers than in the CYP3A5 non-expressers, because CYP3A5 expressers should have high CYP3A5 expression and activity. Likewise, regarding *ABCB1* polymorphisms, we expected the prednisone C_{max} and AUC_{0-24} to be unchanged like in prednisolone or to be lower in the *ABCB1 3435 CC* Carriers than in the *ABCB1 3435 CT/TT* Carriers, because Pg-p production and activity is higher in *ABCB1 3435 CC* Carriers. However, the results were not as expected. The small sample size is likely to be a key limiting factor in the current study. Hence, a larger number of patients need to be studied to confirm the impact of *CYP3A5* and *ABCB1 3435* polymorphisms on prednisone pharmacokinetics. To our knowledge, our data constitute the first report on the pharmacokinetics of prednisone as an immunosuppressive agent describing the influence of *CYP3A5*, *ABCB1 3435* and *POR*28* genetic polymorphisms. Further studies would be helpful to understand the effects of these polymorphisms on prednisone plasma concentrations. *CYP3A4*22* gene polymorphisms were not included in this study because of the low frequency of this genotype in our population. Ethnic factors and patients' sex were shown to have no influence on prednisone C_{max} and AUC_{0-24}.

Prednisone is a well-known substrate of CYP3A and/or P-gp. Effect of prednisone on tacrolimus concentration in renal transplant patients should be taken into consideration. Anglicheau et al. (2003a) found that tacrolimus dose requirement was greatly affected by prednisone dose. The higher the prednisone dosage used, the higher the tacrolimus dosage required to achieve target trough blood concentrations in these patients. The interaction also occurs even with low prednisone dosage. This is due to enzymatic induction of CYP3A and/or P-gp by glucocorticoid. These findings were confirmed by Park et al. (2009) who found an inverse correlation between prednisone daily dose and

tacrolimus exposures. Prednisone dose reduction was associated with an increase in tacrolimus drug exposure. Similarly, a more recent study demonstrated that during the first 6 months after transplantation, prednisone dose significantly influenced tacrolimus blood concentration. Prednisone tacrolimus interaction has more effect on male than female patients (Velickovic-Radovanovic et al., 2012). Differential generation of prednisone in CYP3A5 expressers may influence the prednisolone/tacrolimus interaction.

Chapter 9. General Discussion & Conclusions

Tacrolimus has a narrow therapeutic window of blood concentration and wide variation between individuals in blood concentration achieved by a given dose which is a matter of concern in the clinical practice. Therefore, determination of the optimal tacrolimus dose is necessary to minimize the undesired side effects of tacrolimus while maintaining its efficacy, especially in the early period after transplantation. Different oral formulations of tacrolimus are available and another once daily formulation, Envarsus has recently been given marketing authorisation in the UK. Advagraf® is once-daily tacrolimus with prolonged-release characteristics compared to the initially authorised preparation, Prograf® which has immediate-release absorption profile (Soto et al., 2015). Generic twice-daily tacrolimus preparations that are bioequivalent to Prograf® are now available. A body-weight-based dose of tacrolimus results in a marked variation between individuals in tacrolimus blood concentration. A number of factors are considered to contribute to low and variable bioavailability of tacrolimus, including extensive first pass metabolism and activity of the drug-efflux pump, p-glycoprotein. Tacrolimus is a substrate of CYP3A4, CYP3A5, and Pg-p, which are proteins expressed by *CYP3A4*, *CYP3A5*, and *ABCB1 3435* genes (Hebert, 1997). Hence differences in the level of expression and the bioactivity of these proteins may contribute to the individual variations of tacrolimus pharmacokinetics.

In this study, Advagraf® showed a comparable pharmacokinetic profile to twice-daily tacrolimus confirming that once-daily tacrolimus, Advagraf® is bioequivalent to twice-daily tacrolimus preparations according to the FDA guidelines; 80-125% (FDA., 2003). It also met the bioequivalence acceptance criteria of the European Medicines Agency for tacrolimus; (90-111%) for AUC and (80-125%) for C_{max} (EMA, 2015). We confirmed the repeatedly reported strong correlation between AUC_{0-24} and C_0 for immediate and modified release tacrolimus indicating that measurement of C_0 is appropriate for therapeutic monitoring of Advagraf®. Our results are in accordance with the previous findings in Phase II studies on Advagraf® showing that a given dose of Advagraf® delivered 90% of the area under the concentration-time curve (AUC)

obtained with Prograf® (EMEA, 2007) . However, the mean tacrolimus ratio for C_{max} following the administration of Advagraf was up to 20% less when compared with Prograf®. They noted that the AUC_{0-24} may need to be monitored to ensure maintenance of similar systemic exposure and they found a good correlation between AUC_{0-24} and C_0 for Advagraf® and Prograf® at steady state, as found in the current study. They also observed less between- and within-subject variability in exposure when compared Advagraf® to Prograf®(EMEA, 2007).

Our data showed that switching from immediate to extended release tacrolimus has an impact on between-patient variability of tacrolimus exposure in this cohort of patients. Advagraf® has less between-individual differences in tacrolimus exposure when compared to immediate release tacrolimus.

In addition, we demonstrated that the between-patient variability in tacrolimus daily dose requirement was related to *CYP3A5*3*, *CYP3A4*22* and *ABCB1 3435* gene polymorphisms in stable kidney transplant recipients, as reported previously, suggesting that the pharmacogenetic assessment of *CYP3A5*3*, *CYP3A4*22* and *ABCB1 3435* genotypes may offer an effective tool for individualizing drug therapy by optimizing tacrolimus dosage for both twice daily tacrolimus and Advagraf®.

It is becoming apparent that all individuals express CYP3A4 and CYP3A4 poor metabolizers are rare. However, CYP3A5 expression varies between different individuals. The *CYP3A5*3* allele reduces CYP3A5 production and results in the loss of hepatic CYP3A5 activity (Hustert et al., 2001, Kuehl et al., 2001). Thus, it has been repeatedly reported that patients with the *CYP3A5*3/*3* genotype (CYP3A5 non-expressers) require lower doses to reach similar dose-normalized tacrolimus trough concentrations than patients carrying at least one *CYP3A5*1* allele (CYP3A5 expressers) (Macphee et al., 2005, Vannaprasaht et al., 2013, Ferraris et al., 2011). In our study, we clearly could confirm this effect. Several studies have examined the effect of P-gp on tacrolimus exposure, and conflicting results have been obtained. Some studies reported no correlation between *ABCB1 3435* genotypes and tacrolimus dose and pharmacokinetics (Haufroid et al., 2004, Jun et al., 2009, Vannaprasaht et al.,

2013). However, other studies displayed a significant influence of *ABCB1 3435* genotypes on tacrolimus pharmacokinetics and dose requirements (Zheng et al., 2003, Lopez-Montenegro Soria et al., 2010, Yu et al., 2011), which is in line with our findings. In addition, upon evaluation of *CYP3A5*3* and *ABCB1 3435* genotypes in combination, significant differences in tacrolimus pharmacokinetics were evident between *ABCB1 3435* polymorphisms in CYP3A5 expressers suggesting that *ABCB1 3435* genotype is an important factor in tacrolimus pharmacokinetics particularly in the case of CYP3A5 expressers. These findings support previous findings by Loh and colleagues who reported the same outcome between *ABCB1 3435* and *CYP3A5*3* variants (Loh et al., 2008) and contrast with other studies demonstrating no significant differences in tacrolimus bioavailability between the *ABCB1 3435* polymorphisms in both CYP3A5 expressers and non-expressers (Rong et al., 2010, Tada et al., 2005). Additionally, our data showed the contribution between *CYP3A4*22* polymorphisms and tacrolimus pharmacokinetics confirming the findings of the recently published studies (Elens et al., 2011a, Tavira et al., 2013, Kurzawski et al., 2014).

Interestingly, we found that the influence of *CYP3A5*, *CYP3A4* and *ABCB1 3435* genotypes on tacrolimus exposure was the same for once- and twice daily tacrolimus. No significant difference was observed between these polymorphisms and tacrolimus pharmacokinetics and dose requirements in both tacrolimus preparations.

It has been reported that CYP3A expression reduces progressively along the length of the gut. However, the level of cellular expression of Pg-p increases continuously along the gut length (Thorn et al., 2005). We hypothesised that the influence of these genotypes would apply differently between tacrolimus formulations. Our assumption was that CYP3A5 polymorphisms may have less effect on the oral bioavailability of extended release tacrolimus formulation, Advagraf® which is mostly absorbed lower down the gut than the immediate release preparations of tacrolimus such as Prograf® and Adoport® that are absorbed in the upper part of the gastrointestinal tract (GIT), mainly around the stomach and proximal small intestine (MacPhee, 2012). Our data showed that the impact of *CYP3A5*, *CYP3A4* and *ABCB1 3435* polymorphisms and their combinations had no clear difference between twice-daily tacrolimus and Advagraf®.

This is in accordance with the recently published studies finding that tacrolimus exposure was significantly higher in CYP3A5 non-expressers than in CYP3A5expressers and the degree of difference was similar between Prograf® and Advagraf® (Benkali et al., 2010, Glowacki et al., 2011b, Niioka et al., 2012, Wehland et al., 2011). This may indicate a dominant effect of the liver CYP3A5 on the first-pass metabolism of tacrolimus and a minor influence of intestinal enzymes in tacrolimus metabolism. However, earlier studies in liver transplant recipients have revealed the influence of the intestinal CYP3A5 on tacrolimus absorption. A study by Uesugi et al indicates that intestinal CYP3A5, as well as hepatic CYP3A5, plays an essential role in the first-pass metabolism of orally administered tacrolimus in liver transplantation (Uesugi et al., 2006). Another study in liver transplantation recipients found that tacrolimus pharmacokinetics is mainly influenced by the intestinal CYP3A5 and P-gp expression during the first week; after that, it is mostly affected by the hepatic metabolism (Goto et al., 2004). This can be explained by the minor effect of the intestinal CYP3A5 on tacrolimus metabolism that only appears in the absence of the liver CYP3A5 enzymes. It is also possible that the gradient of CYP3A5 and P-gp expression along the length of the gut was over-estimated in previously published reports (Thorn et al., 2005).

Moreover, this study showed that switching from immediate to extended release tacrolimus formulations did not make any significant difference in WPV of dose-normalized C_0 in both tacrolimus preparations. Similar observations were made in other conversion studies (van Hooff et al., 2012, Wehland et al., 2011, Shuker et al., 2014). However, other studies showed that conversion from Prograf® to Advagraf® was associated with a significantly lower WPV of Tac C_0 (Wu et al., 2011, Alloway et al., 2005). Our findings showed that neither patients treated with twice daily tacrolimus nor patients treated with once- daily tacrolimus show any significant association between WPV of dose-normalized Tac C_0 and *CYP3A5* genotype. This in line with previous published studies (Pashaee et al., 2011, Ro et al., 2012, Wu et al., 2014). The balance of published evidence suggests that conversion from twice daily tacrolimus to Advagraf® is unlikely to impact significantly on WPV in routine renal transplantation.

Additionally, in our data, we found that in spite of being a known substrate of CYP3A and P-glycoprotein (Anglicheau et al., 2003a), prednisolone pharmacokinetics were not associated with *CYP3A5*3* and *ABCB1 3435* polymorphisms. Prednisone behaved differently and *CYP3A5*3* and *ABCB1 3435* genotypes were strongly associated with prednisone pharmacokinetics. CYP3A5 expressers had higher concentrations of prednisone, presumably reflecting preferential metabolism of prednisolone to prednisone. It is worth noting that patient sex had a significant effect on prednisolone C_{max}. Ethnic factors and tacrolimus dose were shown to have no influence on the C_{max} and AUC_{0-24} of prednisolone and prednisone. This indicates that neither the genetic factors nor ethnicity can predict prednisolone plasma concentration. Given the wide variation between individuals in prednisolone blood concentration achieved by a dose of 5 mg prednisolone daily, it may actually be appropriate to consider using TDM, in particular for patients with efficacy failure or toxicity.

Moreover, our study showed that 4β-OHC concentration increased significantly in *CYP3A5*1* allele carriers compared to recipients having *CYP3A5*3*3* genotype (Diczfalusy et al., 2008, Suzuki et al., 2014). The 4β-OHC/C ratio was significantly correlated with tacrolimus exposure and dose requirement. 4β-OHC/C ratio may be a useful biomarker for tacrolimus dosing in renal transplanted patients. While the effect of *CYP3A5*3* genotype and CYP3A activity measured by plasma 4β-OHC/C ratio on tacrolimus exposure were closely linked, they were both found to be independent predictors and would be additive in developing an algorithm for predicting optimal initial tacrolimus dose.

Of note, tacrolimus dose requirement may be modified and its pharmacokinetics can be affected by several parameters including genetic and non-genetic factors. In order to secure the optimal tacrolimus administration, both genetic and non-genetic factors must be taken into account. Most studies search for the genetic polymorphisms that affect the response of individuals to tacrolimus. However, non-genetic factors may have an influence in tacrolimus pharmacokinetics and dose requirements. Hence, we tried to model tacrolimus kinetics based on both genetic and non-genetic factors.

In the present study, we demonstrated that *CYP3A5*3* genotype is a key factor in the prediction of tacrolimus blood concentrations and dose requirement. Many studies highlighted the influence of CYP3A5 in tacrolimus pharmacokinetics and dose requirement and reported that CYP3A5 could be useful to predict the optimal tacrolimus dose (Niioka et al., 2015, Thervet et al., 2008, Birdwell et al., 2015). In a randomized controlled study, kidney transplant recipients receiving tacrolimus doses according to the CYP3A5 genotype reached the target C_0 significantly earlier than recipients used a standard regimen. Although more patients were within the desired tacrolimus target range early after transplantation, a considerable proportion of patients still did not have tacrolimus C_0 levels within the target range points (Thervet et al., 2010) indicating that *CYP3A5* genotype alone is unlikely to be sufficient for successful individualisation of initial tacrolimus dose. Another study found no association between pharmacogenetic adaptation of tacrolimus daily dose and earlier achievement of the tacrolimus target exposure range. No improvement in the clinical outcome was observed (Shuker et al., 2015). In addition to *CYP3A5*3* genotype, our results also confirm a minor role of the *ABCB1 3435* variant allele. This supports previous studies showing a weak association between *ABCB1 3435* polymorphism and tacrolimus dose requirements (Li et al., 2006a, MacPhee et al., 2002, Anglicheau et al., 2003b). However, other studies failed to identify such an association (Tsuchiya et al., 2004, Haufroid et al., 2004, Quteineh et al., 2008, Shi et al., 2013). The reason for the discrepancies between these studies is unclear, but may be due to the food, the genetic effect of other genes, or studies lacking sufficient statistical power.

Moreover, we studied some non-genetic factors, including age, sex, haematocrit, ethnicity, diabetic status, steroid therapy, donor type, time since transplantation and tacrolimus formulation. We found that the donor type, time since transplantation and tacrolimus formulations had no significant effect. Although sex had a significant effect on tacrolimus dose in univariate analysis, this effect was diminished in multivariate regression analysis. Stratta et al reported that sex differences affect tacrolimus dose requirements (Stratta et al., 2012).

Furthermore, we demonstrated that the haematocrit value was strongly correlated with tacrolimus dose, consistent with previous reports showing the influence of haematocrit values in tacrolimus blood concentration (de Jonge et al., 2012, Stratta et al., 2012). Tacrolimus is extensively bound to FK-binding proteins in red blood cells. Hence the haematocrit plays an important role in tacrolimus pharmacokinetics and may need to be considered in tacrolimus dosage regimens especially with significant changes in their levels. In multivariate analysis, diabetic status was significantly associated with tacrolimus dose requirements, confirming previous findings by Chitnis et al who found that diabetic patients have significantly higher dose adjusted tacrolimus blood concentrations compared to non-diabetic patients (Chitnis et al., 2013)**.** Our findings confirm the effect of age on tacrolimus dose requirement. Younger patients required higher tacrolimus dose than older patients. This is in accordance with previous findings indicating the strong correlation of age with tacrolimus dose in both adults (Kim et al., 2012) and paediatric patients (Gijsen et al., 2011). We also demonstrated that ethnicity had a significant effect on tacrolimus dose, black patients required higher tacrolimus dose than white and Asian subjects, which is consistent with previous findings (Macphee et al., 2005). However, the *CYP3A5*3* polymorphism cannot be replaced by ethnicity to predict the tacrolimus dose requirement, even though they are strongly linked.

In this study, different tacrolimus formulation has no influence on tacrolimus dose either in univariate or multivariate analysis. This means that factors were reported to influence tacrolimus dose including age, sex, ethnicity, haematocrit, diabetic status, corticosteroid treatment and *CYP3A5*3/ ABCB1 3435* polymorphisms are the same for both tacrolimus formulations. From our study prediction of tacrolimus dose can be achieved from the following equation:

Dose (mg/kg) = 0.2199 - 0.000622 * Age - 0.1636 * Haematocrit - 0.0387 (if Asian) - 0.0217 (if White) - 0.02665 (if diabetic) + 0.01043 (if treated with corticosteroids) + 0.00974 (if female) + 0.0017 (if *CYP3A5*1/*1/*1/*3 /ABCB1CT/TT* genotype) - 0.0457 (if *CYP3A5*3/*3 /ABCB1CC* genotype) - 0.0534 (if *CYP3A5*3/*3 /ABCB1CT/TT* genotype).

Our findings suggest that taking all these aforementioned factors into consideration may account for 59.9% of the between-individual variability in tacrolimus dose requirements. However, the impact of other factors, including different diet habits, comorbidity and concomitant treatment schemes could not be estimated. These findings may have potential clinical application for initiation and adjustment of tacrolimus therapy. Given the modest impact, if any, of using *CYP3A5* genotype to predict the optimal initial dose of tacrolimus, it may now be appropriate to test algorithms including genetic and non-genetic factors as described here. As a first step, it would be useful to test the predictive value of this equation in an independent group of transplant recipients. Demonstration of clinical utility of an algorithm would require a clinical trial statistically powered to demonstrate improvement in hard clinical endpoints.

Conclusion

The findings of the studies presented have demonstrated that the between-patient variability in tacrolimus daily dose requirement was related to *CYP3A5*3*, *CYP3A4*22* and *ABCB1 3435* gene polymorphisms and that *CYP3A5 *3* genotype is a key factor in the prediction of tacrolimus blood concentrations and dose requirement. Furthermore, the influence of *CYP3A5*3*, *CYP3A4*22* and *ABCB1 3435* genotypes on tacrolimus exposure was the same for once- and twice daily tacrolimus. *CYP3A5*3* polymorphism cannot be replaced by 4B-OHC to predict tacrolimus dose requirement, even though they are strongly linked. Moreover, our data showed that switching from immediate to extended release tacrolimus has an impact on between-patient variability of tacrolimus exposure in this cohort of patients. On the other hand, conversion from immediate to extended release tacrolimus did not make any significant difference in WPV of dose-normalized C_0 and *CYP3A5*3* genotype had no impact on within-patient variability of tacrolimus clearance in once- and twice-daily tacrolimus formulations. In addition to the genetic factors associated with tacrolimus dose, some non-genetic factors, including age, ethnicity, haematocrit, diabetic status and steroid treatment seems to have an influence in tacrolimus dose. This can be applied for both tacrolimus formulations.

Chapter 10. Future Options

Based on the data presented in this thesis, I would propose the following follow-on work.

Development of an algorithm to predict the optimal initial tacrolimus dose based on the genetic polymorphisms and non-genetic factors is worthy of further investigation. To test, this would require a large number of patients to deliver sufficient statistical power. Proposed next steps include:

1) **A retrospective study to validate a predictive equation for initial tacrolimus dose in an independent cohort of renal transplant recipients**

In an independent cohort of transplant patients, tacrolimus dose will be estimated based on *CYP3A5*3/ ABCB1 3435* polymorphisms, age, ethnicity, haematocrit, 4β-hydroxycholesterol /cholesterol ratio, *CYP3A4*22* and compared to the actual tacrolimus dose that achieved target blood concentration. The relationship defined in **Chapter 6** page 228 will be used in this study.

2) **Testing of a population pharmacokinetic model in a prospective clinical trial**

A population pharmacokinetic model for tacrolimus will be developed on basis of the theoretical expectation of a relationship between tacrolimus dose and *CYP3A5*3/ ABCB1 3435* polymorphisms, age, ethnicity, haematocrit, 4β-hydroxycholesterol /cholesterol ratio, diabetic status and steroid therapy being the same for the prolonged release preparation Advagraf® as for the immediate release preparation, Prograf® or Adoport®. The clinical trial will need to show that the combination of genetic and non-genetic factors to tacrolimus dose requirement would improve patient outcome based on measurable clinical endpoints including incidence of acute rejection and NODAT.

The expectation following the identification of a number of gene polymorphisms involved in tacrolimus metabolism is to achieve a better immunosuppressive therapy for

each individual patient with less rejections or adverse effects. However, even if patients are genotyped for gene polymorphisms involved in tacrolimus absorption and metabolism, still a large percentage of tacrolimus dose variability remains unclear. For example, tacrolimus pharmacokinetics is strongly correlated with the expression and activity of the metabolizing enzyme CYP3A5. CYP3A5 expressers required 2-fold higher tacrolimus dose than non-expresser. Two recent clinical studies showed either no association between *CYP3A5* adaptation of tacrolimus daily dose and earlier achievement of the target tacrolimus exposure range (Shuker et al., 2015) or some improvement in the number of patients reaching the target concentration with a considerable proportion of patients outside the target range (Thervet et al., 2010). The Liver is the main site of metabolism of tacrolimus. Detection of CYP3A5 expression helps to explain 30-35% of tacrolimus variability. It cannot give an accurate prediction of tacrolimus trough concentration and dose requirement. While 4β-hydroxycholesterol /cholesterol ratio provided a measure of systemic CYP3A activity, it adds relatively little to the predictive value of CYP3A5 genotype. An alternative approach that has been used to measure systemic CYP3A activity employed midazolam as a drug probe as an adjunct to genotyping (de Jonge et al., 2012).

3) Individualisation of steroid therapy

In this study, we found variation between patients in prednisolone and prednisone blood concentration achieved by 5 mg prednisolone daily, confirming the recent findings demonstrating large within- and between-individual variabilities in prednisolone and prednisone pharmacokinetics, indicating the need for personalizing dosing of glucocorticoids (including prednisolone and prednisone) in organ transplantation (Sagcal-Gironella et al., 2011, Saeves et al., 2012). Interestingly, this study showed a significantly lower concentration of prednisolone metabolite, prednisone in CYP3A5 non-expressers, suggesting that the other main metabolite of prednisolone, 20β-dihydroprednisolone could be produced more than prednisone in the case of CYP3A5 non-expresser. A clinical trial is needed to identify the pharmacodynamics of this metabolite and to see if it is responsible for any of prednisolone adverse effects. Furthermore a TDM clinical trial is also required to find the basis of a definable

relationship between prednisolone dose and plasma concentration and consequently between plasma concentration and the therapeutic effect in order to optimize the clinical outcomes in patients. Therefore, the dose can be adjusted to reach the target therapeutic concentration.

4) **Do the factors that predict pharmacokinetics for Advagraf® apply to Envarsus®**

Different oral formulations of tacrolimus are available. Generic once daily formulation, Envarsus® has recently been given marketing authorisation in the UK. Each formulation of tacrolimus has different excipients which are considered the inactive ingredients that do not have therapeutic role. Some studies have revealed that excipients can cause various side effects (Strauss and Greeff, 2015). Excipients can affect the pharmacokinetics of drugs through their effects on drug absorption (Andrysek, 2006). Therefore, a clinical study is required to study the influence of the pharmacogenetic differences in this new formulation and to study the effect of switching stable renal transplant patients from a twice daily formulation of tacrolimus (Prograf® or Adoport®) or once daily formulation (Advagraf®) to the new once daily formulation (Envarsus®) on within patient variability in pre-dose blood tacrolimus concentration.

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Appendices

Appendix 1. Patient Information Sheet and Consent Form

St George's University of London

St George's Healthcare NHS
NHS Trust

Patient Information Sheet

The influence of *CYP3A5* and *ABCB1* genotype on the pharmacokinetics of twice daily tacrolimus and Advagraf

Introduction and Invitation
We are approaching you about participation in a research project because you have a kidney transplant and are on treatment with tacrolimus. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take the time to read the following information carefully and discuss it with relatives, friends and your GP if you wish. Ask us if there is anything that is not clear or if you would like more information. Take your time to decide whether or not you wish to take part.

Part 1 tells you the purpose of this study and what will happen to you if you take part.
Part 2 gives you more detailed information about the conduct of the study

PART 1
What is the purpose of the study?
Tacrolimus is the main drug used to prevent rejection of kidney transplants. Individual patients require differing doses of Tacrolimus to achieve the target blood concentrations. Recently, we have identified a blood test that determines the presence of genes that influence the amount of tacrolimus that is absorbed into the blood-stream after taking the drug orally. Individuals with this gene may benfit from a higher initial dose of tacrolimus. A new once daily preparation of tacrolimus called Advagraf has become available recently. On average, Advagraf leads to very similar amounts in the blood to Prograf. We do not know whether the genes that influence the absorption of tacrolimus preparations taken twice daily such as Prograf affect the absorption of once daily Advagraf in the same way. The genetic tests have potential value in selecting the correct starting dose of tacrolimus for individual patients.

Why have I been invited to take part?
You will recently have been offered the option of changing your tacrolimus treatment from a twice daily form to the new once daily preparation (Advagraf).

Do I have to take part?
It is up to you to decide. We will describe the study and go through this information sheet, which we will then give to you. We will then ask you to sign a consent form to show you have agreed to take part. You are free to withdraw at any time, without giving a reason. This would not affect the standard of care you receive.

What will happen to me if I agree to take part?
DNA will be prepared from a 5 mL sample of blood (equivalent to one teaspoon) and used to determine the type of gene that you posess for genes that influence the uptake and clearance of immunosuppressive drugs from the body. These genes are called *CYP3A5, ABCB1, CYP3A4 and P450 oxidoreductase*. The results of this genetic test may take

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several weeks to come back. You will be asked to attend the hospital at least 7 days before starting the study to collect specifically labelled study medication. The study involves having a series of blood samples collected over a 24 hour period while you are taking your routine twice daily dose of tacrolimus. Your treatment will then be changed to once daily Advagraf and two weeks later a further series of blood samples will be collected over a 24 hour period. The blood samples will be collected in St. George's Hospital. A venous cannula will be placed into the hand or arm for blood sampling and will remain in place throughout the sampling period. Blood samples (5mL, equivalent to one teaspoon) will be collected prior to taking the morning drug dose at 08:00 am and then at the time points indicated in table 1. This will require a 24 hour stay for the first set of samples with the option to go home between the 20:00 and 08:00 samples on the second sampling day. Accommodation will be provided. Subjects will be required to fast for 2 hours before taking the drug dose and for 1 hour afterwards.

Table 1: blood sampling schedule

Time point (hrs)	Twice daily tacrolimus	Advagraf	Actual time
0	*	*	08:00
0.5	*		08:30
1	*	*	09:00
2	*	*	10:00
3		*	
4	*	*	12:00
5			
6	*	*	14:00
7			
8	*	*	16:00
9			
10		*	18:00
11			
12	*	*	20:00
12.5	*		20:30
13	*		21:00
14	*		22:00
15			23:00
16	*		24:00
17			
18			
19			
20			
21			
22			
23			
24	*	*	08:00

Concentrations of tacrolimus, prednisolone and an enzyme called 4β hydroxycholesterol will be measured in the blood samples.

Expenses incurred in attending for these studies will be reimbursed in addition to a payment of £100 in recognition of the time given up by the subject to participate in the study.

What are the benefits of taking part in this research project?
This study may help us to use Tacrolimus in the treatment of renal transplant patients more effectively in the future. There will not be any direct clinical benefit to you as a result of participating.

Are there any risks?
The only potential risks are associated with insertion of the venous cannula for blood sampling. There may be pain or bruising at the site of cannula insertion and a very small risk of infection.

What if there is a problem?
Any complaint about the way you have been dealt with during the study or any possible harm you might suffer will be addressed. The detailed information on this is given in Part 2.

What happens after the research has stopped?
Following collection of the blood samples, participation in the project is over. The results of the research will be reviewed and published in scientific and medical journals. If you would specifically like a copy of the results you can contact one of the researchers (details below) and request the information.

Will taking part in the study remain confidential?
Yes. All information which is collected about you during the course of the research will be kept strictly confidential. It will be stored in password protected computers within the hospital or Medical School and only accessed by authorised members of the research team.

Contact Details
Please see below for the contact details of the investigators

This completes Part 1. Please take the time to read Part 2 before making any decision

PART 2
What happens if I want to withdraw from the Study?
You may withdraw from the study at any point, for any reason, with no requirement to provide an explanation. After you have given the blood samples you can contact us at a later date to say you no longer wish to help us with our project and we will destroy samples.

What if there is a problem?
Complaints
If you have a concern about any aspect of this study, you should ask to speak with the researchers who will do their best to answer your questions (details below)
If you remain unhappy and wish to complain formally, you can do this through the NHS Complaints Procedure. Details can be obtained from the hospital.
You can also contact Patient Advisory Liaison Service (PALS), tel 0208 725 0453,
Harm
If you are harmed by taking part in this research project, there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for it. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms should be available to you. Please contact Patient Advisory Liaison Service (PALS) at St Georges Hospital

Will my taking part in this study be kept confidential?
Yes. All information which is collected about you during the course of the research will be kept strictly confidential. We will use information from your medical records and the computer data base. It will be stored in password protected computers within the hospital or Medical School. This can only be accessed by authorised members of the research and clinical team. All data will be stored for 10 years. Once the study is completed the data will be archived to protect it.

Involvement of your GP
We will inform your GP that you have participated in this study.

What will happen to any samples I give?
The samples are coded and your name removed to prevent identification. The samples will be stored until full study results have been published. If you withdraw from the study we will destroy these samples if you wish. The initial blood sample will be used to prepare DNA to determine the type of gene that you posess for genes that influence the uptake and clearance of immunosuppressive drugs from the body. The serial blood samples collected over 24 hour periods will be used to measure changes in the concentration of tacrolimus and prednisolone in the blood over time.

Will genetic tests be done?
Yes: testing will be performed on genes that influence the uptake and clearance of immunosuppressive drugs from the body. The genetic testing will not predict risk to yourself of any disease and has no implications for your relatives.

What will happen to the results of the study?

The results of the study will be provided to you, on request. The results will be published in peer-reviewed journals.

Who is organising and funding the research
The research is being organised by the Renal Group in the Division of Clinical Sciences, St. George's, University of London. The study 'Sponsor' is St. George's, University of London. The study has been funded by a research grant from Astellas Pharmaceuticals.

Who has reviewed the study?
All research in the NHS is looked at by an independent group of people, called a Research Ethics Committee to protect your safety, rights, wellbeing and dignity. This study has been reviewed and given a favourable opinion by the London-West London Research Ethics Committee.

You will be given a copy of this information sheet and a signed copy of the consent form.

Thank you for taking time to read this

Dr Iain MacPhee, Reader and Consultant in Renal Medicine,
Division of Clinical Sciences: Renal Medicine,
St. George's, University of London,
Cranmer Terrace,
London SW17 0RE.

0208 725 5035

St George's University of London

St George's Healthcare NHS
NHS Trust

Patient Identification Number:
Research and Ethics Committee Number: 09/H0707/91
Version 4: (3rd October 2012)

Consent Form

The influence of *CYP3A5* and *ABCB1* genotype on the pharmacokinetics of Prograf and Advagraf

Please Initial box

Name of Researcher: Dr Iain MacPhee

1. I confirm that I have read and understand the information sheet dated 3rd October 2012 (version: 6) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected ☐
3. I understand that relevant sections of my medical notes and data collecting during the study, may be looked at by responsible individuals from St. George's, University of London/ St George's Healthcare NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records ☐
4. I agree to my G.P. being informed of my participation in this study ☐
5. I agree to take part in the above study ☐

Name	Date	Signature
Name of Person taking consent	Date	Signature
Researcher	Date	Signature

When completed, 1 for patient; 1 for researcher site file; 1 (original) to be kept in medical notes

Appendix 2. Case Report Form

St George's University of London

ADVAGRAF

St George's Healthcare NHS
NHS Trust

Short Title	Pharmacokinetics of twice daily Tacrolimus and Once daily Tacrolimus		
Sponsor	St George's University of London		
Eudra CT number	2009-013461-25	R&D number	09.0098
Chief Investigator	Dr Iain MacPhee		

CASE REPORT FORM

Site Name: ☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐

Site Number: ☐☐

Subject Number: ☐☐☐

- I am confident that the information supplied in this case report form is complete and accurate
- I confirm that the study was conducted in accordance with the protocol and any protocol amendments
- I confirm that written informed consent was obtained prior to study entry

Investigator's Signature: _____

Date of Signature (dd/mm/yyyy): _____

Subject Number:	

INFORMED CONSENT

Please note written informed consent must be given before any study specific procedures take place or any current therapy is discontinued for the purposes of participation in this study.

	Yes	**No**
Has the subject freely given written informed consent?		

Date of Consent given d d m m y y y y

Eligibility criteria:

INCLUSION CRITERIA			
		Yes	No
1	Renal transplant patient at least 6 weeks after transplantation		
2	Aged at least 18 years old		
3	On Tacrolimus twice a day with planned change in treatment to once daily Tacrolimus as part of standard of care		
4	Signed and dated consent obtained before screening and before performance of any protocol-specific tests		
Answers to questions 1 – 4 must be ' YES ' to qualify for the study			

EXCLUSION CRITERIA – Patients with potent P450 3A and P-glycoprotein inducers or inhibitors			
INDUCERS			
		Yes	No
1	Carbamazepine		
2	Phenytoin		
3	Rifampicin		
4	Any other inducer		
Answers to questions 1 – 4 must be ' NO ' to qualify for the study			
INHIBITORS			
		Yes	No
1	Diltiazem		
2	Erythromycin		
3	Fluconazole		
4	Verapamil		
5	Any other inhibitor		
Answers to questions 1 – 5 must be ' NO ' to qualify for the study			

Name ..... **Signature**.....

Job Title.....

Subject Number:	

Screening

Date of Assessments d d m m y y y y

Ethnic group:

Caucasian	
Black African	
Black Caribbean	
South Asian	
Other (please specify) :	

DEMOGRAPHIC DATA

DOB: d d m m y y y y

Age (yrs): Sex: Female

Height (m): • Male

Weight (kgs): •

VITAL SIGNS

Pulse rate bpm

Blood pressure (seated) / mmHg

Original renal disease: _____

MEDICATIONS TAKEN

Is the subject currently or previously taking any medication including OTC, vitamins and/or supplements?

Yes No

** Record all medication on Concomitant Medications Page (see below)*

St George's University of London

Name ..... Signature.....

Job Title.....

Subject Number:	

CONCOMITANT MEDICATIONS

Medication	Dose	Frequency	Units	Indication	Start Date (dd/mm/yyyy)	Stop Date (dd/mm/yyyy)	Ongoing

Note: Please use additional page if required.

Name .....

Signature.....

Job Title.....

Subject Number:	

PREVIOUS MEDICAL HISTORY

Is there any relevant medical history in the following systems?

Code	System	Yes*	No		Code	System	Yes*	No
1	Cardiovascular				9	Neoplasia		
2	Respiratory				10	Neurological		
3	Hepato-biliary				11	Psychological		
4	Gastro-intestinal				12	Immunological		
5	Genito-urinary				13	Dermatological		
6	Endocrine				14	Allergies		
7	Haematological				15	Eyes, ear, nose, throat		
8	Musculo-skeletal				00	Other		

**If YES for any of the above, enter the code for each condition in the boxes below; give further details (including dates) and state if the condition is currently or potentially active. If giving details of surgery, please specify the underlying cause. Use a separate line for each condition.*

		Currently active?	
Code	**Details** (including dates)	**Yes**	**No**

Name ..... **Signature**.....

Job Title.....

Subject Number:	

SCREENING

Does the patient have routine laboratory tests for the following Haematology or biochemistry from the most recent outpatient/clinic visit to the planned visit of the first PK sampling?

Haematology:

Date sample collected: d d m m n y y y y

Test name	Value	Units	Ranges
HB		g/l	120 to 160
WBC		10^9/L	4.0 to 11.0
PLATELET		10^9/L	150 to 450
MCV		fl	80 to 97
NEUTROPHIL		10^9/L	1.7 to 8.0
LYMPHOCYTE		10^9/L	1.0 to 4.0
MONOCYTE		10^9/L	0.24 to 1.1
EOSINOPHIL		10^9/L	0.1 to 0.8
BASOPHIL		10^9/L	0.0 to 0.3
HCT		%	0.41 to 0.52
RBC		10^{12}/L	4.5 to 6.0
MCH		pg	27 to 33
MCHC		g/dl	32 to 36.5
RDW		%	11.5 to 14.5
%Hypo RBC		%	

Are there any clinically significant results? Tick Yes ☐ No ☐

If yes, Comment:

Name ..... Signature.....

Job Title.....

Subject Number:	

Screening

Biochemistry:

Date sample collected d d m m y y y y

Test name	Value	Units	Ranges
SODIUM		mmol/L	133 to 146
POTASSIUM		mmol/L	3.5 to 5.3
CHLORIDE		mmol/L	95 to 108
BICARBONATE		mmol/L	22 to 29
UREA		mmol/L	2.5 to 7.8
CREATININE		umol/L	60 to 110
eGFR		mL/min/1.73m²	
Coded Comment For Above Test: Multiply eGFR by 1.21 If Afro-Caribbean. Guidance: see RCGP leaflet or www.emrn.org.uk			
GLUCOSE		mmol/L	3.0 to 6.0
Coded Comment For Above Test: Fasting glucose (3.0 - 6.0 mmol/L)			
BILIRUBIN		umol/L	0 to 21
ALANINE TRANSAMINASE		U/L	0 to 52
ALKALINE PHOSPHATASE		U/L	30 to 130
ALBUMIN		g/L	35 to 50
GAMMA GT		U/L	0 to 64
CALCIUM		mmol/L	2.15 to 2.50
PHOSPHATE		mmol/L	0.80 to 1.50
ADJUSTED CALCIUM		mmol/L	2.2 to 2.6
C REACTIVE PROTEIN		mg/L	0.0 to 10.0

Are there any clinically significant results? Tick Yes ☐ No ☐

If yes, Comment:

_____

St George's University of London

Name ..... **Signature**.....

Job Title.....

Subject Number:	

End of Visit Checklist: to be completed by Investigator

	Yes	No
1. Does the subject satisfy the inclusion and exclusion criteria to date?	☐	☐
2. Have all screening procedures been completed?	☐	☐
3. Has the concomitant medication page been completed?	☐	☐
4. Is the subject willing to proceed?	☐	☐

Investigator

	Yes	No
Is the subject to continue?	☐	☐
Subject received sufficient medication until arranged treatment change date	☐	☐
Have the dosing instructions been explained to the Subject?	☐	☐
Is the Subject aware of travelling to St George's for the pharmacokinetic sampling?	☐	☐

Name ..... **Signature**.....

Job Title.....

Subject Number:	

VISIT 1: PHARMACOKINETIC PROFILE (1)

On treatment with twice daily Tacrolimus

Date of visit | d | d | m | m | n | y | y | y | y |

Prescribed total daily dose .....

Dose of Tacrolimus AM ..... mg PM ..... mg

	Capsule count	Batch Number	Manufacturer
☐	5mg x.....	_____	.....
☐	1mg x.....	_____	.....
☐	0.5mg x.....	_____	.....

Subject completed diary and returned: Yes ☐ No ☐

If no, Please provide reason_____

_____

Name ..... Signature.....

Job Title.....

Subject Number:	

Visit 1

PHYSICAL EXAMINATION

Date of Physical Examination: d d m m y y y y

Name	Comments
General appearance	
Head & Neck	
Ears, Eyes, Nose &Throat	
Respiratory	
Chest	
Cardiovascular	
Gastrointestinal	
Abdominal	
Neurological	
Lymphatic	
Skin	
Musculoskeletal	
Allergies	
Genito urinary	
Other	

Name ..... **Signature**.....

Job Title.....

Subject Number:	

Visit 1
Day 0

Sampling times:

Time point (hrs)	Prograf	Advagraf	Actual time	Time collected	Person taking sample	COMMENTS
0	*	*	08:00			
0.5	*		08:30			
1	*	*	09:00			
2	*	*	10:00			
3		*				
4	*	*	12:00			
5						
6	*	*	14:00			
7						
8	*	*	16:00			
9						
10		*	18:00			
11						
12	*	*	20:00			
12.5	*		20:30			
13	*		21:00			
14	*		22:00			
15			23:00			
16	*		24:00			
17						
18						
19						
20						
21						
22						
23						
24	*	*	08:00			

✶ Samples stored as per protocol: **Yes** ☐ **No** ☐

IF NOT PLEASE ENTER IN THE PROTOCOL DEVIATION LOG

Name ..... **Signature**.....

Job Title.....

Subject Number:	

DATE OF TREATMENT CHANGE FROM TWICE DAILY Tacrolimus to

ONCE DAILY Tacrolimus

Day 1

Date of treatment Change | d | d | m | m | y | y | y | y |

Dose of Tacrolimus AM ..... mg

Capsule count	Batch Number	Manufacturer
☐ 5mg x.....	_____	.....
☐ 1mg x.....	_____	.....
☐ 0.5mg x.....	_____	.....

Subject completed diary and returned: Yes ☐ No ☐

If no, Please provide reason_____

_____

Name ..... Signature.....

Job Title.....

Subject Number:	

VISIT 3: PHARMACOKINETIC PROFILE (2)
Day 14

On treatment with once daily Tacrolimus

Date of visit | d | d | m | m | y | y | y | y |

Sampling times:

Time point (hrs)	Tacrolimus Twice Daily	Tacrolimus Once Daily	Actual time	Time collected	Person taking sample	Comments
0	*	*	08:00			
0.5	*		08:30			
1	*	*	09:00			
2	*	*	10:00			
3		*	11:00			
4	*	*	12:00			
5						
6	*	*	14:00			
7						
8	*	*	16:00			
9						
10		*	18:00			
11						
12	*	*	20:00			
12.5	*		20:30			
13	*		21:00			
14	*		22:00			
15			23:00			
16	*		24:00			
17						
18						
19						
20						
21						
22						
23						
24	*	*	08:00			

✶ Samples stored as per protocol: **Yes** ☐ **No** ☐

IF NOT PLEASE ENTER IN THE PROTOCOL DEVIATION LOG

Name ..... **Signature**.....

Job Title.....

St George's University of London

Subject Number:	

Has patient completed study: Yes ☐ No ☐

If Yes, End of Study date: ☐☐☐☐☐☐☐☐

If No, Please fill the 'Off study form' overleaf

COMMENTS PAGE

Are there any comments for this patient that is not covered elsewhere in the CRF (For instance, protocol deviations, missed sampling etc.)? NO ☐

Comments	Signature/ Date:

Name ..... Signature.....

Job Title.....

Subject Number:	

OFF STUDY FORM

Date Off Study (dd/mm/yyyy):
Date Last Study Medication Taken(dd/mm/yyyy):

Reason Off Study (Please mark only the primary reason.
Reasons other than **Completed Study** require explanation next to response.

- ☐ Completed Study
- ☐ AE/SAE (complete AE CRF & SAE form, if applicable)
- ☐ Lost to follow up
- ☐ Non-Compliant participant
- ☐ Concomitant medication
- ☐ Medical Contraindication
- ☐ Withdraw Consent
- ☐ Death (complete SAE form)
- ☐ Other

Explanation_____
_____
_____
_____

Name ..... **Signature**.....

Job Title.....

Subject Number:	

Tacrolimus Time and Concentration chart:

No	Time point			Tacrolimus Blood Concentration	
	Protocol	Actual		Visit 1 Twice daily Tacrolimus µg/l	Visit 3 Once daily Tacrolimus µg/l
		Visit 1	Visit 3		
1	08:00				
2	08:30				
3	09:00				
4	10:00				
5	11:00				
6	12:00				
7	14:00				
8	16:00				
9	18:00				
10	20:00				
11	20:30				
12	21:00				
13	22:00				
14	24:00				
15	08:00				

Name ..... **Signature**.....

Job Title.....

Subject Number:	

ADVERSE EVENTS

Has the subject experienced any adverse events since the informed consent was signed? Yes, specify below ☐ No ☐

AE no.	**Adverse Events** (diagnosis (if known) or signs/symptoms)	**Start Date** (dd/mm/yyyy) **and Time** (24 hour clock)	**Stop Date** (dd/mm/yyyy) **and Time** (24 hour clock)	**Outcome** 1 = recovered 2 = recovered with sequelae 3 = continuing 4 = patient died 5 = Change in AE 6 = unknown	**Severity** 1 = Mild 2 = Moderate 3 = Severe	**Plausible relationship to Study Drug** (**Yes/No)	**Action taken with study drug** 1 = None 2 = Dose reduction temporarily 3 = Dose reduced 4 = Discontinued temporarily 5 = Discontinued	**Withdrawn due to AE?** (Yes/No)	**Serious AE? (SAE)** (*Yes/No)	**Expected or **Unexpected?**

Reminder- please ensure all Adverse events are documented in the medical notes

✶ **If yes, please also document and complete JREO SAE form and forward to the JREO via fax 0208 725 0794 or email adverseevents@sgul.ac.uk**

** if SAE AND Plausible relationship AND Unexpected **Report SUSAR immediately to JREO as above**

St George's University of London

Name .....

Signature.....

Job Title.....

Subject Number:	

Has patient completed study: Yes ☐ No ☐

If Yes, End of Study date: ☐☐☐☐☐☐☐☐☐☐

If No, Please fill the 'Off study form' overleaf

COMMENTS PAGE

Are there any comments for this patient that is not covered elsewhere in the CRF (For instance, protocol deviations, missed sampling etc.)? NO ☐

Comments	Signature/ Date:

Name ..... **Signature**.....

Job Title.....

Subject Number:	

OFF STUDY FORM

Date Off Study (dd/mm/yyyy):
Date Last Study Medication Taken(dd/mm/yyyy):

Reason Off Study (Please mark only the primary reason.
Reasons other than **Completed Study** require explanation next to response.

- ☐ Completed Study
- ☐ AE/SAE (complete AE CRF & SAE form, if applicable)
- ☐ Lost to follow up
- ☐ Non-Compliant participant
- ☐ Concomitant medication
- ☐ Medical Contraindication
- ☐ Withdraw Consent
- ☐ Death (complete SAE form)
- ☐ Other

Explanation_____
_____
_____
_____

Name ..... **Signature**.....

Job Title.....

Appendix 3. List of the Concomitant Medications

Concomitant medication	Patients	Concomitant medication	Patients
Adalat La	2	Ismn	1
Alendronic Acid (As Alendronate Sodium)	4	Lansoprazole	5
Alfacalcidol	35	Lantus Insulin	2
Alfuzosin	2	Levimir Insulin	1
Allopurinol	6	Losartan	2
Amlodipine	31	Metoprolol	13
Amoxicillin	2	Metronidazole (Metrotop)	1
Aspirin	19	Minoxidil	1
Atorvastatin	35	Mircera	1
Azathioprine	14	Moxonidine	1
Bendroflumethiazide (Bendrofluazide)	5	Mycophenolate Mofetil	19
Bisoprolol	9	Nifedipine	7
Calcichew	1	Nitrofurantoin	1
Calcitriol	2	Normacol	1
Candesartan	12	Olanzapine	1
Carvedilol	2	Omacor	1
Cefalexin	1	Omeprazole	30
Cefuroxime	1	Paracetamol	1
Cinacalcet	6	Paroxetine	1
Ciprofloxacin	3	Pravastatin	3
Citalopram	1	Prednisolone	39
Co-Amoxiclav	3	Pregabalin	1
Colchicine	1	Pregaday	1
Co-Trimoxazole	4	Propranolol	1
Darbepoetin Alfa (Aranesp)	1	Pyridoxine	3
Dorzolamide Eye Drops	1	Ramipril	16
Doxazosin	20	Ranitidine	1
Esomeprazole	1	Risedronate	1
Ezetimibe	4	Salbutamol	1
Ferrous Sulphate	6	Sildenafil	1
Fluoxetine	1	Sodium Valproate	2
Folic Acid	2	Sotalol	2
Furosemide(Frusemide)	2	Tamsulosin	3

Gliclazide	9	Telmisartan	1
Hepatitis B Vaccine	17	Thyroxine	1
Hydralazine	1	Tibolone	1
Hydroxocobalamin	1	Tramadol	1
Insulin Detemir	1	Trimethoprim	1
Insulin Glargine	2	Valganciclovir	6
Insulin Human Aspart Novomix	9	Venofer 100mg/5ml Intravenous Solution	3
Insulin Novorapide	7	Vitamin D	2
Irbesartan	1	Warfarin	1